

CHARACTERIZATION OF HEMOPOIESIS IN A
LACTATE DEHYDROGENASE DEFICIENT
MOUSE MUTANT Ldh-1^c/Ldh-1^c

TAPATI DATTA

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ABBREVIATIONS

BFU-E	Burst-forming units-erythroid
BMC	Bone marrow cells
BPA	Burst promoting activity
CdR	Deoxycytidine
CFC	Colony-forming cells
CFU-E	Colony-forming units-erythroid
CFU-S	Colony-forming units-spleen
Con-A	Concanavalin A
CSF	Colony-stimulating factor
DDW	Double distilled water
EB	Erythroblasts
EPO	Erythropoietin
FCS	Fetal calf serum
FUdR	Fluorodeoxyuridine
G-CSF	Granulocyte colony-stimulating factor
GM-CFC	Granulocyte-macrophage colony-forming cells
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Hb	Hemoglobin
Hct	Hematocrit
IL-3	Interleukin 3
LD _{50/30}	Lethal dose _{50/30}
LDH	Lactate dehydrogenase
MCGF	Mast cell growth factor
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
M-CSF	Macrophage colony-stimulating factor

MCV	Mean cellular volume
MLCM	Mouse lung conditioned medium
MTPBS	Mouse tonicity phosphate buffered saline
Multi-CFC	Multipotential colony-forming cells
Multi-CSF	Multipotential colony-stimulating factor
PHA	Phytohemagglutinin
PWM	Pokeweed mitogen
RBC	Red blood cells
SCM	Spleen cell conditioned medium
SFDME	Serum free Dulbecco's modified Eagle's medium
SPC	Spleen cells
TBI	Total body irradiation
TdR	Thymidine
TMP	Thymidine monophosphate
WBC	White blood cells
WEHI-3B-CM	WEHI-3B conditioned medium

CHAPTER 1. INTRODUCTION

Investigation of the blood cells is the first traditional approach to find the cause of a hematological disease. In a normal organism, the circulating cells in blood are mostly mature cells and have a limited life span. In order to maintain a steady state within the organism these cells are regularly produced from immature progenitors through a complex process of proliferation and differentiation. This complex process, with its highly organized regulations at the different cellular levels, is called hemopoiesis. Malfunction of proliferation and/or differentiation can ultimately become the cause of a disease. At present, regulation of hemopoiesis is extensively studied at the cellular and molecular level with the aim, among others, to understand the nature of disturbance in hemopoietic malignancies.

Most of our present knowledge about hemopoiesis was derived from studies performed in inbred strains of the species mouse. To offer a short review of hemopoiesis, results derived from studies involving mice will be presented in the following chapter.

CHAPTER 2. FUNDAMENTALS OF HEMOPOIESIS

2.1 The hemopoietic system

2.1.1 Embryonic hemopoiesis

The first site of embryonic hemopoiesis in the life of an organism has been documented in the blood islands of yolk-sac mesoderm. Results of several investigations indicate that the hemopoietic stem cells migrate from the yolk-sac to the body of the embryo, and colonize and proliferate in the liver (Moore and Metcalf, 1970). The liver is the most important hemopoietic organ in the mammalian embryo. In the mouse, liver erythropoiesis starts during the 10th or 11th day of gestation and is followed by megakaryocyte and granulocyte production. Thereafter, hemopoiesis is taken up by the spleen and then by the bone marrow (see review Metcalf and Moore, 1971).

2.1.2 Hemopoietic organization

In the mouse, during postnatal life, liver hemopoiesis begins to diminish within 2 weeks after birth and gradually ceases (for review see Tavassoli and Yoffey, 1983), whereas splenic hemopoiesis continues for the rest of its life. In the adult mouse, the major part of hemopoiesis takes place in the bone marrow, which is composed of hemopoietic cells supported by a specially organized matrix of stromal component. The stromal component is also called hemopoietic microenvironment. The microenvironment is involved in the regulation of self-replication, proliferation and differentiation of hemopoietic cells, and the release

of mature end-cells into the blood stream by cell to cell contact and/or production of humoral factors (Weiss, 1976; Shaklai and Tavassoli, 1979; Weiss, 1980). Lymphopoietic activities are found in the thymus until puberty and for the rest of the postnatal life in the spleen, lymph nodes, Payer's patches, appendix and bone marrow.

Blood cells are mostly mature cells and are derivatives of the hemopoietic cells from bone marrow and spleen. These blood cells, for the sake of convenience, have been divided into three groups: 1) red blood cells (RBC), consisting of erythrocytes with a small fraction of reticulocytes, 2) white blood cells (WBC) consisting of neutrophilic and eosinophilic granulocytes, monocytes, lymphocytes (including thymus- and bone marrow-derived lymphocytes, known as T and B lymphocytes, respectively) and 3) platelets. The morphologically recognizable immature cells of each lineage are also differentiable and named according to their hierarchy, e. g., myeloblasts, promyeloblasts, myelocytes or metamyelocytes for the granulocytic lineage; the erythroid lineage includes proerythroblasts, basophilic erythroblasts (EB), polychromatic EB, orthochromatic EB and reticulocytes. The blood cells and their immature precursors are quite easily distinguishable in a properly stained smear of bone marrow or spleen (T and B lymphocytes can be recognized by applying immunological methods). However, it is often difficult to place immature blast cells in a particular lineage, since they are morphologically very similar to each other.

A family-tree scheme gives us a concept of the hierarchial organization of hemopoiesis (Fig. 1, drawn after Metcalf, 1984 and Walker et al., 1985) and a basic idea of the interrelationships among the different subsets of it. The pluripotent hemopoietic stem cells, also called CFU-S or colony-forming units-spleen (since they can produce

spleen colonies in a lethally irradiated mouse) give rise to progenitor cells of different lineages (Till and McCulloch, 1961; Abramson et al., 1977). These progenitor cells are irreversibly committed to a definite direction and possess probably little or no self-renewal capacity (Metcalf, 1984, 1984a). In a suitable medium in the presence of an appropriate hemopoietic stimulating factor (commonly called colony-stimulating factor or CSF) and under proper cultural conditions progenitor cells can proliferate and differentiate to produce colonies of mature cells, and are therefore termed as colony-forming cells or CFC, using a prefix to designate the lineage.

The different aspects of hemopoietic stem and progenitor cells, and colony-stimulating factors are described below.

2.2 Pluripotent hemopoietic stem cells (CFU-S)

The hemopoietic pluripotent stem cells possess a high degree of self-renewal capacity. The direct evidence of a pluripotent hemopoietic stem cell was shown for the first time by Till and McCulloch (1961). Bone marrow cells of a mouse when injected into a lethally irradiated recipient produced discrete macroscopically visible spleen colonies within 7-12 days. The clonality of these colonies could be proved by the use of specific chromosome markers (Becker et al., 1963; Fowler et al., 1967; Wu et al., 1967; Chen and Schooley, 1968). Cells producing such colonies were termed as colony-forming units-spleen or CFU-S. Histomorphological analysis showed that these colonies apparently contained cells of either one of the 3 lineages (erythroid, granulocytic or megakaryocytic) or a mixture of them (Lewis and Trobaugh, 1964; Fowler et

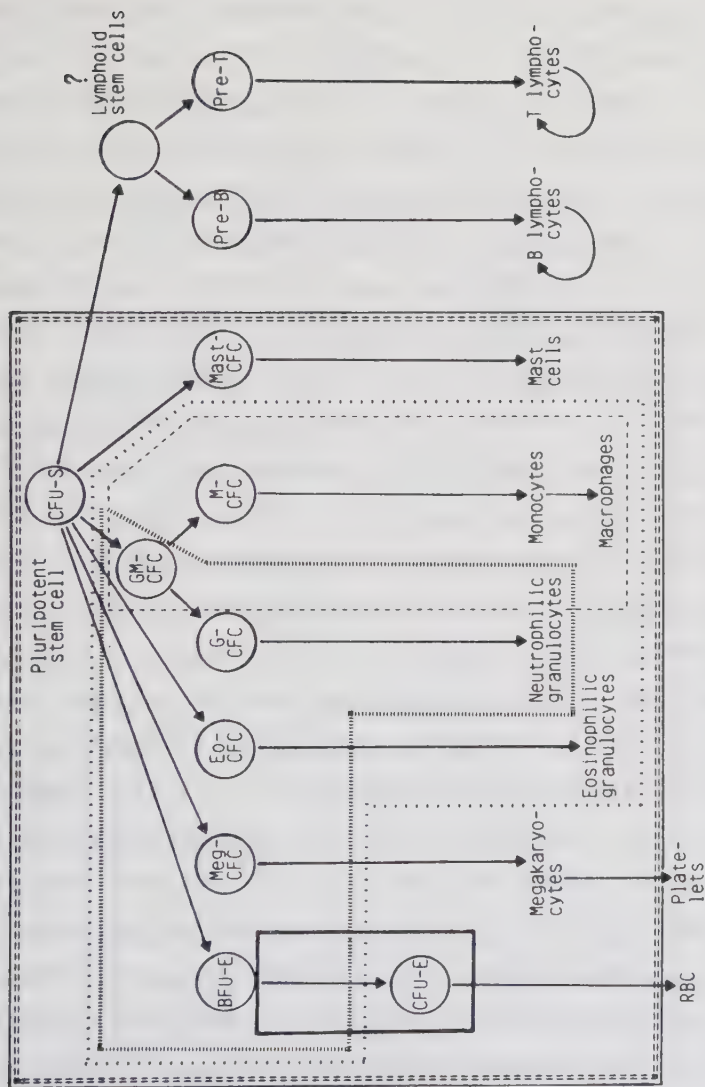


Fig. 1. Schematic representation of the hierarchical organization in hemopoietic system. Each boundary represents a CSF enclosing the cells on which it acts (see text) (after Metcalf 1984; Walker et al., 1985). — IL-3 or Multi-CSF; GM-CSF; --- M-CSF; — EPO.

al., 1967). Abramson (1977) showed that the content of a single colony when injected into a lethally irradiated mouse was able to produce all subtypes of hemopoietic cells including lymphocytes. These results gave enough evidence for the multipotentiality of CFU-S. However, not all the CFU-S do possess the capacity to repopulate an irradiated recipient (Hodgson and Bradley, 1979). Moreover, two different subsets of CFU-S have been identified differing in their self-renewal capacity and their ability to produce progeny. One subset, forming 7-day colonies, possesses relatively low self-renewal capacity and produces a relatively small population of progeny. The other subset, which forms visible colonies after 12 days (Magli et al., 1982; Johnson and Nicola, 1984) has a higher self-replicating, proliferating and differentiating capability. These two subpopulations designated as CFU-S-II and CFU-S-I, respectively, could be separated by the method of fluorescence-activated cell sorting (Metcalf et al., 1983; Johnson and Nicola, 1984). Using a technique developed by Johnson and Metcalf (1977) to clone multipotential colony-forming cells, the Multi-CFC, colonies containing all hemopoietic cells could be recognized (Metcalf et al., 1979; Schrader et al., 1981). Additionally, these colonies may contain several progenitors like Multi-CFC and CFU-S (Metcalf et al., 1979; Humphries et al., 1979; Johnson and Nicola, 1984). Due to the multipotential nature and self-maintaining capability of Multi-CFC, they have been considered by Metcalf (1984) to represent a subset of CFU-S, namely the CFU-S-II. On the other hand, the cells forming blast cell colonies in vitro (Keller and Phillips, 1982; Nakahata and Ogawa, 1982) are supposed to be of the same category as CFU-S-I (Metcalf, 1984).

2.3 Hemopoietic progenitor cells

The pluripotent hemopoietic stem cell (CFU-S) produces committed progenitor cells of different lineages (Fig. 1). It is possible to clone these progenitors by semisolid culture techniques, where they grow as colonies and can be quantified very easily. In the semisolid matrix, which is made by mixing the culture medium with agar or methylcellulose, the progenitors can be immobilized as well as distributed homogeneously. This type of culture technique began first with the development of the technique for cloning the common progenitor of granulocyte and monocyte, the GM-CFC, in semisolid agar medium (Bradley and Metcalf, 1966; Ichikawa et al., 1966).

There are two major classes of erythroid progenitors, which form colonies in culture: 1) the early erythroid progenitors produce colonies having several foci, i.e., burst-like appearance, and are termed as burst-forming units-erythroid or BFU-E; 2) the late erythroid progenitors are called colony-forming units-erythroid or CFU-E. The culture techniques for erythroid progenitor cells came into existence a few years later. In the mouse, the methods to clone the late and early erythroid progenitors, the CFU-E and BFU-E, respectively, were developed by Stephenson et al. (1971) and Axelrad et al. (1973), respectively.

The lymphocytes originate and develop in a different and complex manner. The precursors of T lymphocytes originate in the bone marrow and migrate to the thymus. Through further divisions progenitors of T lymphocytes are produced which may undergo additional proliferation in other lymphatic sites. The B lymphocytes originate from the pluripotent hemopoietic stem cells in the bone marrow and spleen, and can also be

produced from activated B lymphocytes present in the spleen or other lymphoid tissues. Whether a distinct bipotent lymphoid stem cell exists with a capacity to differentiate either along T or B lymphoid lineage is still open. Moreover, using the clonal cultures, which exist up to now for both types of lymphocytes, it is not possible to detect the actual number of progenitors, since many of the mature lymphocytes, when stimulated properly, undergo clonal proliferation. In this context, the regulatory effect of thymocytes and T lymphocytes on the hemopoiesis is worth mentioning. There is strong evidence suggesting that they play an important role at the stem cell (CFU-S) level and also in the differentiation pathway towards erythropoiesis (reviews Eliason, 1984; Zipori, 1984; Goodman and Shinpock, 1984; Lepault et al., 1984; Sharkis, 1984).

All the culture techniques and their practical significance have been described in detail by Metcalf (1984, 1984a). Techniques used in this work will be described under 5.10.

2.4 Regulatory factors of the hemopoietic system --- the colony-stimulating factors (CSFs)

The CSFs regulate the proliferation, differentiation and maturation of hemopoietic cells. These are glycoproteins of molecular weights between 23,000-70,000 (in the mouse). The CSFs have been termed alternatively by different authors using either a prefix or a suffix indicating the lineage. Furthermore, for some CSFs quite different terminologies have been used, e.g., erythropoietin (EPO), interleukin 3 (IL-3) etc., due to a particular historical background. Metcalf (1984, 1984a) has summa-

rized the alternative terminologies used in different laboratories at present.

Since the beginning of the clonal assay in the mid 1960s several CSFs have been extracted from various tissues or from media conditioned by normal or neoplastic cells (Metcalf 1984, 1984a). CSFs are also produced after mitogenic or allogeneic stimulation of lymphocytes (Parker and Metcalf, 1974, 1974a). Moreover, GM-CSF and G-CSF in serum increases 50-100 times when adult mice are injected intravenously with bacterial cell wall products (Metcalf, 1971; Quesenberry et al., 1972; Metcalf, 1980).

In the mouse, the production and function of granulocytes and macrophages are controlled by the interaction of four distinct colony-stimulating factors (Metcalf, 1985, 1986a; Walker et al., 1985) (Fig. 1). Multi-CSF, also known as interleukin 3 (IL-3, Ihle et al., 1982) or mast cell growth factor (MCGF, Ihle et al., 1983), controls the production of most of the mature hemopoietic cells (neutrophilic granulocytes, monocytes, erythroid cells, eosinophils, megakaryocytes and mast cells) from their hemopoietic progenitors and self-replication of hemopoietic stem cells as well (Ihle et al., 1983). Burst-promoting activity (BPA), a regulatory factor for the early erythroid progenitor cells (BFU-E), has also been found to be identical with Multi-CSF (Ihle et al., 1983). GM-CSF stimulates primarily the maturation of granulocytes and macrophages, and to a small extent eosinophils (Johnson and Metcalf, 1980; Metcalf, 1984). GM-CSF supports formation of pure granulocyte and macrophage colonies as well as mixed granulocyte-macrophage colonies. In addition, it can initiate the proliferation of erythroid, megakaryocytic and some multi-potential precursors (Metcalf, 1980). The difference between Multi-CSF and GM-CSF in stimulating ery-

throid progenitors is that the former can regulate the production of fully hemoglobinized colonies, while the latter can influence only a part of the differentiation process, and the presence of EPO is necessary to complete hemoglobinization. M-CSF, also called CSF-1 (Stanley and Heard, 1977), and G-CSF are mostly lineage specific unless used at a high concentration, and act on the progenitors of macrophages and granulocytes, respectively. A small number of granulocyte-macrophage colonies and a few granulocyte colonies may develop after stimulation with M-CSF. G-CSF is known to have a weak effect similar to that of GM-CSF on the early erythroid progenitors (Metcalf, 1984). In the present work, BPA will be used to designate the regulatory factor for BFU-E and IL-3 for that of mast cells, both representing the Multi-CSF.

Erythropoietin (EPO) is another glycoprotein, biochemically distinct from the above four colony-stimulating factors. In vivo action of EPO is comparable to its in vitro regulation of erythropoiesis (see review Metcalf, 1984). EPO has been detected in the kidney, in plasma, urine and fetal liver. Plasma and urine erythropoietin levels increase under anemic conditions caused by bleeding and decreases after hypertransfusion (Lord, 1979). Erythropoietins have been extracted and purified from human (Miyake et al., 1977) and sheep (Goldwasser, 1975) sources. Recently, murine erythropoietin has also been purified (Cutler et al., 1985). Similar molecular weight (40,000 - 45,000) was found for both anemic mouse serum EPO and human urine EPO.

The proliferation of late erythroid progenitor cells (CFU-E) is regulated by erythropoietin. However, using purified EPO no colony formation was found after 7 days (Metcalf and Johnson, 1979), indicating that EPO alone could not support the growth of progenitor cells like BFU-E and Multi-CFC (Metcalf et al., 1980). In the erythroid

pathway, proliferation and differentiation of early erythroid progenitor cells (BFU-E) are regulated by BPA.

In the past years, scientists in many laboratories have tried to clone the genes coding for all known CSFs including EPO, by which enough material to test their biological action in vivo is provided and an eventual clinical application becomes feasible. The recombinant CSFs cloned up to now have been summarized by Metcalf (1986). In vivo action of recombinant Multi-CSF, GM-CSF or EPO showed results comparable to their in vitro actions.

2.5 Hemopoietic microenvironment

The interaction between hemopoietic cells and the elements of the supporting stroma, in the bone marrow or spleen, plays an important role on the resulting hemopoiesis. The extravascular compartment in the bone marrow where hemopoiesis takes place is called the hemopoietic cord, which is supported by scaffolding of stromal cells, namely reticular cells and macrophages. In the hemopoietic cord itself distinct compartments for different lineages of hemopoiesis have been observed (see review Tavassoli and Yoffey, 1983).

Dexter et al. (1977) have established an in vitro culture system to maintain the stromal elements in culture flasks as an adherent layer which supports the self-maintenance, proliferation, differentiation and maturation of hemopoietic cells and turns out to be a unique system to study the stroma and hemopoietic cell interactions (see review Dexter, 1979). The major part of this in vitro stroma is the fibroblast cells corresponding in vivo to the reticular cells (Tavassoli and

Takahashi, 1982). The stroma cells can be grown as fibroblast colonies (CFU-F) in culture and quantified (Friedenstein et al., 1970, 1974; Friedenstein, 1976). However, in these colonies macrophages and hemopoietic cells may be present which adhere to the fibroblasts.

CHAPTER 3. DISCOVERY OF THE LACTATE DEHYDROGENASE (LDH) MUTANT MOUSE AND PRELIMINARY OBSERVATIONS

A mouse mutant with lactate dehydrogenase (LDH) deficiency was discovered in the offspring population following treatment of (101/ElxC3H/El)F₁ male mice with the mutagen procarbazine hydrochloride. The animals were immediately mated with test stock females (Pretsch and Charles, 1980; Charles and Pretsch, 1981) possessing seven recessive markers (Ehling, 1978). Electrophoretical analysis of the liver extract showed a banding alteration of LDH-A, the form which predominates in the skeletal muscle. Consequently, the authors concluded that the mutation affected the locus coding for LDH-A. The first detected mutant was heterozygous, and homozygotes were obtained by crossing heterozygous mice. After 12 generations of backcrossing with the inbred strain C3H/El the mutants were considered to have a uniform genetic background of C3H/El (Green, 1966, 1981). The mutation is designated with the allele symbol LDH-1^C, the wild type being LDH-1^a. Table 1 shows the LDH activities in different tissues of the three genotypes (Pretsch and Charles, 1987). It is obvious that the mutant LDH activities are much lower in those tissues in which LDH-A predominates, e.g., blood, liver, muscle, spleen, than in other tissues where mostly the other type, LDH-B, is present. The mutation is codominantly inherited since the heterozygotes show LDH values of about 50% of the wild type.

Table 2 shows some biochemical characteristics in the serum of the mutants (Pretsch and Charles, 1987). The serum bilirubin level is increased moderately in the heterozygous mutant and is twice the wild type value in the homozygous mutants. The value of iron is decreased in the homozygous mutants. Uric acid values are almost the same in all

Table 1. LDH activities in several tissues from homozygous wild type (Ldh-1^a/Ldh-1^a), heterozygous mutant (Ldh-1^a/Ldh-1^c), and homozygous mutant (Ldh-1^c/Ldh-1^c) mice^a

Tissue	<u>Ldh-1^a/Ldh-1^a</u>	<u>Ldh-1^a/Ldh-1^c</u>	<u>Ldh-1^c/Ldh-1^c</u>
Liver	2 811 ± 615 (100 ± 22)	1 940 ± 196 (69 ± 7)	262 ± 69 (9 ± 3)
Muscle	14 025 ± 1 642 (100 ± 12)	10 907 ± 1 406 (78 ± 10)	2 181 ± 615 (16 ± 4)
Spleen	968 ± 183 (100 ± 19)	855 ± 142 (88 ± 15)	85 ± 78 (9 ± 8)
Lung	635 ± 88 (100 ± 14)	498 ± 60 (78 ± 9)	144 ± 29 (23 ± 5)
Heart	2 992 ± 558 (100 ± 19)	2 970 ± 506 (99 ± 17)	1 635 ± 580 (55 ± 19)
Kidney	3 010 ± 403 (100 ± 13)	2 593 ± 392 (86 ± 13)	1 385 ± 275 (46 ± 9)
Brain	2 656 ± 851 (100 ± 32)	1 754 ± 493 (66 ± 19)	1 256 ± 446 (47 ± 17)
Testes	1 046 ± 72 (100 ± 7)	963 ± 124 (92 ± 12)	869 ± 118 (83 ± 11)

^aData are expressed as mean ± SD of 5 mice (in case of testes 2). LDH activities are expressed as IU/mg protein ($\times 10^3$). Percentages of normal are given in parenthesis (Pretsch and Charles, 1987).

genotypes.

Table 2. Some biochemical parameters in the serum of homozygous wild type (Ldh-1^a/Ldh-1^a), heterozygous mutant (Ldh-1^a/Ldh-1^c), and homozygous mutant (Ldh-1^c/Ldh-1^c) mice^a

Parameters	<u>Ldh-1^a/Ldh-1^a</u>	<u>Ldh-1^a/Ldh-1^c</u>	<u>Ldh-1^c/Ldh-1^c</u>
Bilirubin (mg/dl)	0.57 ± 0.10 (100 ± 18)	0.73 ± 0.14 (128 ± 25)	1.14 ± 0.15 (200 ± 26)
Iron (µg/dl)	334 ± 54 (100 ± 16)	337 ± 30 (101 ± 9)	229 ± 56 (69 ± 17)
Uric acid (mg/dl)	2.91 ± 0.29 (100 ± 10)	2.92 ± 0.40 (100 ± 14)	3.21 ± 0.30 (110 ± 10)

^aData are expressed as mean ± SD of 4 wild type, 6 heterozygous mutant and 3 homozygous mutant mice. Percentages of normal are given in parentheses (Pretsch and Charles, 1987).

Apart from the greatly diminished activity of the muscle type LDH, further phenotypic expressions of the LDH mutation are represented by a low number (50% of wild type mice) of red blood cells (RBC), low hemoglobin concentration, an extremely increased reticulocyte fraction, an elevated bilirubin level and splenomegaly. These features tally with the clinical manifestations of hemolytic anemia suggesting a shortened erythrocyte life span. In spite of this hemolytic disease the homozygous mutants turned out to have a largely normal life span and fertility; they could be paired and the litter size was normal. Several mutations causing hemolytic anemia in the mouse have been reported, the alleles of which are as follows: sph, sph^{ha} (formerly: ha), sph^{2Bc}, ja and nb (Russel, 1979; Bernstein, 1980; Unger et al., 1983). A deficiency of the erythrocyte membrane protein spectrin has been documented and no change could be found in the glycolytic pathway (Lux et al., 1981; Unger et al., 1983). In contrast to the LDH mutant, their life span and fertility were reduced (Russel, 1979).

The homozygous LDH-mutant with its steady state of hemopoiesis in the presence of a high rate of red cell loss offers a unique model to study the mechanisms of hemopoietic compensation. The experimental strategy was based on the following biological questions:

1. How does the LDH deficiency affect hemopoietic cells at different stages of differentiation, especially in the erythroid pathway?
2. What is the nature of the compensatory mechanism in this mutant?
3. What is the role of the enlarged spleen in the hemopoietic compensation?
4. What would be the effect of an additional stress (e.g., irradiation)

on the hemopoiesis of the mutants?

5. Are the stem cells of the wild type mice capable of proliferating in the hemopoietic environment of the mutants and vice versa, and is it possible to cure the hemolytic anemia through bone marrow grafting?

Quantification of morphologically recognizable hemopoietic compartments as well as of the multipotent hemopoietic stem cells (CFU-S), granulocyte-macrophage colony-forming cells (GM-CFC), and erythroid early (burst-forming units, BFU-E) and late (colony-forming units, CFU-E) progenitors was performed, thereby addressing the question at which compartmental level compensation for hemolytic anemia occurs. The cell cycle status of the above hemopoietic cells and proliferation kinetics of the morphologically recognizable erythroid cells were analysed for their contribution to the erythroid compensation. The importance of the spleen was judged with the help of splenectomy experiments. Hemopoietic changes in the anemic mice followed by irradiation and radiosensitivity of the different hemopoietic cells as well as of the whole mouse were investigated. Bone marrow transplantation experiments were carried out to produce wild type/homozygous mutant and homozygous mutant/wild type chimeras with the aim to assess the stem cell repopulating qualities and to cure the hemolytic disease. Heterozygous mutants were considered only for baseline studies and excluded from other investigations, since they showed almost no difference from the wild type mice.

CHAPTER 5. MATERIALS AND METHODS

5.1 Experimental animals

Ten to fourteen week-old mice of both sexes and of the genotypes homozygous mutants (Ldh-1^C/Ldh-1^C), heterozygous mutants (Ldh-1^a/Ldh-1^C) and homozygous wild type (Ldh-1^a/Ldh-1^a) mice were used. For the sake of convenience, in the present work, +*/+*, +/+* and +/+ will be used for those three genotypes, respectively. The mice were obtained from the Institute of Genetics, GSF. Other mouse strains, such as C57Bl/6 and C3H, were available from the central breeding facility of the GSF. Animals were kept in microlon boxes, fed with commercial dry pellets and tap water ad libitum. The room temperature in animal quarters was maintained at 23(±2)°C with 65(±10)% humidity and room ventilation. The day and night rhythm was not influenced artificially.

5.2 Collection of peripheral blood and counting of different blood cells

Peripheral blood was collected from the orbital sinus of mice anesthetized with ether. For a small quantity (100-300 µl) of blood, heparinized 100 µl micropipettes (Brand, Wertheim, FRG) were used. A micropipette was carefully inserted at a definite angle under the eyeball through the conjunctiva of the medial canthus into the orbital sinus (Cunliffe-Beamer, 1983). Bleeding was stopped instantaneously after withdrawal of the micropipette. Larger quantities of blood were collected in EDTA-coated tubes (Greiner, Nürtingen, FRG) by bleeding the

mice from the orbital sinus followed by subsequent sacrifice of the animals by cervical dislocation.

50 μ l of blood sample were mixed with 950 μ l of Turk's solution for half a minute. An improved Neubauer hemocytometer (Resistance, FRG) was filled with the mixed sample. White blood cells (WBC) within 4 big squares were counted and the number was multiplied by 50 to obtain the value per microliter of blood according to Hallmann (1980).

In order to perform differential count of WBC, air-dried blood films were fixed with absolute methanol for 25 min, again air-dried and stained with a 7% solution of Giemsa (Merck, FRG) in phosphate buffer, pH 7.2 (Puffer nach Weise, Merck), for 20 min. Finally they were washed in the same phosphate buffer, air-dried and mounted with Eukitt. At least 250 nucleated cells were counted from each blood sample. The morphological criteria were based on the description by Schermer (1967).

For counting of red blood cells (RBC) 20 μ l of blood sample were mixed with 3980 μ l of Hayem's solution for 1-2 min. Cells within 5 medium-sized squares of a hemocytometer were counted and the number was multiplied by 10^4 to acquire the value per microliter of blood. The method was adapted from Hallmann (1980).

The percentage of reticulocytes was determined by mixing blood samples in equal proportion with a 1% solution of brilliant cresyl blue (Merck) in 0.9% NaCl. It was kept for 10 min, mixed again and smeared on clean fat-free slides. At least 1000 RBC were counted under oil immersion of a Zeiss microscope (Carl Zeiss, FRG) and the percentage of reticulocytes was determined. Instructions given by Hallmann (1980) were followed.

With help of a calibrated eyepiece the diameters of erythrocytes and

reticulocytes (200 of each) were measured for wild type and homozygous mutant mice. The slides prepared for reticulocyte counting were used for this purpose.

In order to determine the hemoglobin concentration (Hb, g/dl), two different solutions were used according to the procedure described in the manufacture's (Boehringer, Mannheim, FRG) handbook. Hemoglobin was transformed into hemoglobin cyanide by potassium cyanide and potassium hexacyanoferrat-(III). The color intensity of the transformed hemoglobin cyanide is proportional to the hemoglobin concentration and is measured at 546 nm in an automatic analyzer (ACP 5040, Eppendorf, Hamburg, FRG).

For hematocrit determination (Hct, %) blood samples were taken carefully into a micro-hematocrit tube, one end of which was sealed with plastocine, and placed into a Adams micro-hematocrit centrifuge (Becton, Dickinson & Co., USA).

Further parameters were derived as follows:

$$\text{Mean cell volume (MCV, } \mu\text{m}^3) = (\text{Hct} / \text{RBC} \times 10^6 / \mu\text{l}) \times 10$$

$$\text{Mean cell hemoglobin (MCH, pg)} = (\text{Hb} / \text{RBC} \times 10^6 / \mu\text{l}) \times 10$$

$$\text{Mean cell hemoglobin concentration (MCHC, g/dl)} = (\text{Hb} / \text{Hct}) \times 100$$

5.3 Preparation of bone marrow and spleen smears, their staining and differential counts

Mice were killed with cervical dislocation. The skin was removed and the femur freed from the surrounding muscles. The femur shaft was split with a pair of scissors. With the help of a narrow painting brush, slightly wetted in serum, the bone marrow was taken out and smears were

drawn in a serpentine fashion.

The same mice as above were used for the preparation of spleen. Skin and muscle over the spleen were removed. The spleen was separated carefully and cut into pieces. One piece was held with the forceps and smears were drawn by the cut end in a serpentine fashion.

Smears were air-dried and stained with Giemsa by the same method as used for blood films. Criteria for the different morphologically recognizable cells were based on the description by Miller and Osmond (1974). At least 500 bone marrow and spleen cells were counted for each mouse.

5.4 Determination of LDH activity in peripheral blood, bone marrow and different organs

LDH activity was measured in the Institut für Säugetiergenetik, GSF. The method has been described by Charles and Pretsch (1987).

Whole blood samples were kept at 4°C until use. To lyse the cells each sample was diluted (1:11) with cold 0.1 M triethanolamine, 1mM EDTA buffer, pH 7.5 (TRAB buffer) containing 0.005% saponine and 5mM dithiothreitol (DTT) and allowed to stand for 10 min at 4°C. The homolysates were centrifuged at 8,800 g for 10 min at 4°C, the supernatants containing the soluble proteins were diluted (1:2) with cold TRAB buffer containing 5mM DTT. This 1:22 diluted solution was finally used.

Each sample of bone marrow cells and other organs was mixed with distilled water and homogenized with an Ultra-turrax homogenizer (Braun, Melsungen, FRG) at a rate of 20,000 rpm for 0.5 min. The homolysates were centrifuged at 100,000 g for 10 min. The supernatants

with soluble proteins were taken for final measurements without dilution.

The assay was adapted from the methods of Bergmeyer and Bernt (1974) and Beutler (1975). LDH was determined in an automatic enzyme analyzer (ACP 5040, Eppendorf, Hamburg, FRG). 0.1 M imidazole, 70 mM KCl, 18 mM $MgSO_4$ buffer, pH 7.0 (IMI buffer) was used. As substrate 8.5 mM pyruvate (Na salt) and as coenzyme 0.28 mM NADH (Na_2 salt) were used. The assay was done at 25°C and the activity was determined from the decrease of NADH indicated by the changes in absorbance at 334 nm. 250 μ l buffer, 100 μ l starting reagent (including substrate, coenzyme) and depending on the activity of LDH in the sample, 5-50 μ l of homolysate or organ extract were pipetted into the cuvetts. The enzyme activities were expressed by the analyzer as IU/ml. One unit of enzyme activity is defined as the amount of enzyme utilized during the conversion one μ mole of substrate per minute under the specific conditions of the procedure.

In whole blood samples RBC were counted and LDH activity was calculated as IU/ 10^9 RBC. For BMC it was expressed as IU/ 10^9 cells. For all other sample it was expressed as IU/g protein. Protein content was determined according to a modified method of Lowry (Bergmeyer et al., 1974).

5.5 Preparation of cell suspensions

5.5.1 Preparation of bone marrow cell suspension

Mice killed by cervical dislocation were disinfected with 70% alcohol.

A 21 gauge needle fitted on a 1 ml syringe containing cold mouse tonicity phosphate buffered saline (MTPBS) (pH 7.3, phosphate salt 0.02 M, NaCl 0.15 M) or alpha medium (Gibco, Scotland) was kept ready. The skin was removed and femur freed from the surrounding muscles with the help of sterilized instruments. The femur was cut at its proximal and distal end. The needle was drilled carefully into the open proximal end and the content was injected with slight pressure to eject the marrow into a sterile disposable plastic tube with a cap (Greiner, FRG). The bone cavity was washed gently several times by aspiration and ejection. A single cell suspension was prepared from the collected bone marrow by repeated gentle pipetting with 5 and 1 ml pipettes, respectively. Afterwards it was kept on ice until use.

5.5.2 Preparation of spleen cell suspension

Mice were killed by cervical dislocation and disinfected with 70% alcohol. Skin and muscle over the spleen were removed, the spleen separated carefully and placed in sterile medium as mentioned above. It was cut into small pieces with a sterile pair of scissors and passed through a sterile stainless steel sieve with the help of a glass piston, followed by repeated pipetting to get a single cell suspension. Remaining clots were removed.

5.6 Viable cell counting

The eosin dye exclusion technique was applied. A cell suspension was mixed 1:10 with an eosin solution (0.2% eosin and 15% fetal calf serum

in 0.9% NaCl). The viable nucleated (v.n.) cells not taking up eosin were counted by use of a hemocytometer and their number per ml was determined. Erythrocytes and dead cells were not counted.

5.7 Preparation of conditioned media

5.7.1 Mouse lung conditioned medium (MLCM)

Mouse lung conditioned medium was prepared following the method of Sheridan and Metcalf (1973) and used as a source of GM-CSF. C57Bl/6 mice were injected intravenously with 0.2 ml of MTPBS containing 5 µg of lipopolysaccharide (LPS of E.Coli 0111:B4 W, Difco laboratories, Detroit, USA). Three hours later the mice were killed. Each pair of lung was excised intact and rinsed 3 times in saline, and finally transferred into a plastic culture tube (Greiner, FRG) containing 5 ml serum free Dulbecco's modified Eagle's medium with 5mM lithium chloride. The tubes were kept at 37°C under fully humidified atmosphere with 10% CO₂. After two days the conditioned medium was filtered through a gauze, pooled and partially purified with ammonium sulphate (55% and 85%) and subsequently dialysed against MTPBS for 72 h. Each MLCM charge was tested and a dilution at the level of plateau stimulation (twice the minimum needed for optimum stimulation of colony growth in semisolid agar culture) was selected. The MLCM was stored at -20°C.

5.7.2 WEHI-3B-conditioned medium (WEHI-3B-CM)

The WEHI-3B cells derived from a murine myelomonocytic leukemia cell

line had been obtained from the Walter and Eliza Hall Institute, Melbourne, Australia. WEHI-3B-CM was used as a source of burst-promoting-activity (BPA) (Iscoe and Sieber, 1975). 1×10^6 WEHI-3B cells were cultured in serum-free RPMI 1640 medium (Gibco, Grand Island, New York) supplemented with L-glutamine (2 mM), α -thioglycerol (10^{-4} M), penicillin-streptomycin (100 units/ml), bovine serum albumin (BSA) 1%, human transferrin (280 μ g/ml) fully saturated with FeCl_3 and a sonicated mixture of lipids containing synthetic L- α -dipalmitoyl lecithin (10^{-5} M), oleic acid (2×10^{-5} M) (Iscoe et al., 1980). The supernatants were harvested on day 5 and the optimum concentration for BFU-E (burst-forming-units erythroid) growth in semisolid methylcellulose culture medium was selected, according to the same principle as described for MLCM. The conditioned medium was stored at -20°C .

5.7.3 Spleen cell conditioned medium (SCM)

Standard SCM was prepared according to the method of Metcalf and Johnson (1978). 2×10^6 spleen cells (SPC) of C57Bl/6 mice in RPMI 1640 medium (Seromed, Biochrom, Berlin, FRG) were stimulated with 5% of a freshly prepared (the content of one bottle was dissolved in 75 ml of distilled water) pokeweed mitogen (PWM) (Gibco, Scotland, GB) solution. The medium contained 5% fetal calf serum (FCS), 1% penicillin-streptomycin, 0.5% L-glutamine and 5% PWM solution. 200 ml cell suspension was incubated in 500 ml Erlenmeyer flask for 7 days at 37°C in a fully humidified atmosphere with 10% CO_2 in air. The harvested conditioned medium was centrifuged at 8,000 g for 20 min to remove the cells and cell debris. The supernatant was tested for BPA (or Multi-CSF) activity.

Spleen cell conditioned media produced by spleen cells of wild type and homozygous mutant mice were compared for their CSF activity. SCM was prepared by incubating spleen cells (1×10^6 , 2×10^6 , or 4×10^6) of both genotypes (pool of 3 spleens) with three different lectins, either concanavalin A (Con A, Sigma Chemical Co., St. Louis, USA), or phytohemagglutinin (PHA, Wellcome Research Laboratories, Beckenham Kent, England), or PWM (see above). Different concentrations of the lectins (PHA 1% or 2%, PWM 5% or 10%, Con A 2.5, 5, 10 or 20 $\mu\text{g/ml}$) were tested in pilot studies for this strain of mice (data not shown). The response of spleen cells in culture was found to be similar in both genotypes and the optimum concentrations were selected. The concentration of PWM was the same as above, and those of Con A and PHA was 5 $\mu\text{g/ml}$ and 1% (of the solution prepared according to the manufacturer's instructions), respectively. Controls were performed without any lectin. The composition of the medium was the same as described above for SCM. Conditioned media were prepared in 24-well plates in triplicate for each group, and harvested and pooled on days 3, 5 and 7.

5.8 Quantification of interleukin 3 (IL-3) or Multi-CSF activity in SCM

The proliferation of mast cells (a factor dependent murine mast cell line developed in our laboratory was used) stimulated with SCM was determined as a measure of IL-3 activity. Mast cells ($2 \times 10^5/\text{ml}$, 100 $\mu\text{l/well}$) were stimulated with SCM for 72 h in quadruplicate cultures using 96-well plates. Proliferation was measured using a colorimetric method described by Mosmann (1983). The basic principle is that the

pale yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide of Sigma) is converted by active mitochondria to a dark blue formazan product, which can be measured photometrically. In this test the total mitochondrial activity stands for the total number of cells, and has indeed been shown that both parameters run in parallel (Mosmann, 1983; Baumgarten, 1985). 10 µl of a 5 mg/ml MTT solution were added to each well containing SCM-stimulated mast cells and the plates were incubated at 37°C for 4 h. In order to dissolve the dark blue crystals, 100 µl/well of acid-isopropanol (0.04 N HCl in isopropanol) were added and thoroughly mixed by means of a multichannel pipette. Plates were read with a Micro-ELISA-reader (SLT-labinstruments, Austria) using a test wave length of 565 nm and a reference wave length of 670 nm.

5.9 Spleen colony assay for hemopoietic stem cells (CFU-S) and determination of seeding factor f

The spleen colony assay for CFU-S according to the method of Till and McCulloch (1961) was performed for the quantification of CFU-S as well as to determine the radiosensitivity of CFU-S. Recipient mice were female C3H/El and 6 mice were taken per group. Following lethal irradiation (see 5.15.1) an appropriate number of bone marrow cells (BMC) or spleen cells (SPC) in 0.5 ml MTPBS was injected into the lateral vein of each recipient. Before injection the mice were warmed under an infra-red lamp for 5-15 min so that the expanded veins would be easier to find. The number of cells to be injected was adjusted in preliminary experiments to avoid confluency of spleen colonies (data not shown). On

day 10 or 12 the recipients were killed, and the spleens were removed and fixed in Bouin's solution. The spleen colonies, produced by the CFU-S present in BMC or SPC, were counted with the naked eye. In each experiment 6 irradiated mice received no cells, in order to evaluate the endogenously formed colony number.

Only a small fraction, f , of the CFU-S in the injected cell suspension forms colonies in the spleen of the recipient mouse and the rest resides elsewhere, e.g., in the bone marrow, and cannot be counted by this technique. In order to calculate the actual number of CFU-S present in a cell suspension one should know the value of f , which is used to designate the seeding efficiency. This was determined by a modified method of Siminovitch et al. (1963):

f = number of CFU-S recovered from a cell suspension with a known number of CFU-S, divided by the total number of CFU-S in the cell suspension

20×10^6 viable nucleated BMC (pool of 6 mice) were injected intravenously (i.v.) into 4 primary recipients. 24 h later their spleens were removed, suspended, and the equivalent of 1/10th of a spleen in 0.5 ml was injected into each of 8 secondary recipients. The CFU-S recovered could be evaluated from these 8 secondary recipients. The total number of CFU-S injected into each of the secondary recipients could be calculated from the number of spleen colonies produced by the BMC. This was determined in the same way as described above, taking 10 recipients and injecting 10^5 BMC/mouse.

5.10 Culture of hemopoietic progenitor cells in semisolid media

The semisolid culture systems provide the opportunity to clone hemopoietic precursor cells, which in the presence of an appropriate colony stimulating factor will proliferate and differentiate in a definite direction to form colonies after a definite period of time. The semisolid culture systems prove to be applicable for two different measurements: 1) quantification and characterization of hemopoietic precursor cells, 2) detection and quantification of colony stimulating factors (Metcalf, 1984). In the present studies semisolid culture systems were used for both purposes.

5.10.1 Semisolid agar culture system

Pre-tested (see 5.7.1) charges of MLCM were used routinely as a source of GM-CSF. Appropriate dilution of MLCM in 0.1 ml volume was prepipetted in each of the 3-5 replicas of 35 mm Petri dishes (Greiner, Nürtingen). Double strength serum free Dulbecco's modified Eagle's medium (SFDME) was mixed with a pre-tested fetal calf serum (FCS) in a 6:4 ratio. The composition of the SFDME was as follows: 10.0 g Dulbecco's modified Eagle's medium (powder) (Gibco Limited, Paisley, Scotland); 390 ml double distilled water (DDW); 3 ml L-asparagine (6.7 mg/ml) (Merck, FRG); 1.5 ml DEAE-dextran (Pharmacia Fine Chemicals AB, Uppsala, Sweden); 1% of 10,000 units/ml penicillin-streptomycin (Gibco limited); 4.9 g sodium bicarbonate (NaHCO_3). The medium was filtered through a sterile filter and stored at 4°C. Agar medium was prepared by mixing equal parts of 0.6% bacto agar (Difco Laboratories, Detroit, USA) and of the above mixture of SFDME and serum. Weighed amounts of

agar powder were boiled in DDW for 2 min and cooled down to 50°C shortly before use. By mixing the warm agar solution with the cold medium a temperature appropriate for the cells was reached. Cell suspension in a minimum volume (not larger than 0.8 ml for 4 ml of agar medium), with a concentration of cells producing about 100 colonies in culture, was added to the agar medium, mixed well and aliquotes of 1 ml were pipetted into each of the Petri dishes already containing 0.1 ml of stimulus. Each aliquot was mixed carefully with the stimulus. The agar medium was then allowed to gel at room temperature to immobilize the cells in it. The cultures were incubated for 7 days at 37°C in a fully humidified atmosphere containing 10% CO₂.

GM-CFC colonies were scored routinely at day 7 using a dissecting microscope (Olympus, Tokyo, Japan) with indirect light at a 32-fold magnification. Cell aggregates containing at least 50 cells were scored as colonies.

5.10.2 Semisolid methyl cellulose culture system

This culture system was used to grow both BFU-E and CFU-E colonies following the method of Iscove and Sieber (1975) and keeping 3-5 replicas per group. Double strength alpha medium was prepared by mixing MEM (minimum essential medium) alpha medium (Gibco) with 500 ml DDW; 2.2 g of NaHCO₃ and 10 ml of penicillin-streptomycin were then added. A 2% stock solution of methylcellulose was prepared in the following way: 22 g methocel (Fluka, Switzerland) were added to 500 ml of boiling DDW in an Erlenmeyer flask, boiled gently for 2 min, and allowed to cool down to 45°C while continuously being stirred with a magnet. 500 ml of double strength alpha medium were added to this solution and the flask

was kept at 4°C for 24 h, again under continuous stirring. The solution was then divided into several 50 ml Falcon tubes (Becton Dickinson & Co., USA) and frozen at -20°C until needed.

Pre-tested charges of WEHI-3B-CM (see 5.7.2) or SCM (see 5.7.3) were used as a source of BPA. It was prepipetted in a volume of 0.2 ml per plate. In addition to BPA, the BFU-E cultures contained 1.5 units of erythropoietin (EPO step III, Connaught, Willowdale, Canada), whereas the CFU-E cultures were stimulated by 1 unit of EPO, a concentration at which maximum stimulation was achieved (Fig. 3). EPO was dissolved in alpha medium and stored according to the manufacturer's instruction (stored frozen and used within 4 weeks). The stimuli were prepipetted in a total volume of 0.3 ml per plate. The culture medium was composed of 0.8% methyl cellulose (0.4 ml of 2% stock solution), 20% FCS, 10^{-4} M α -thioglycerol (Serva, Heidelberg, FRG) and 1% BSA (Fluka). The optimum contrations of the different components were tested in preliminary experiments (data not shown). Cell suspension (0.04 ml/plate) was added to the culture medium just before plating and mixed with a syringe fitted with a 1.4 x 40 mm needle. 0.7 ml aliquots were added into each Petri dish containing 0.3 ml of stimuli and mixed carefully. The cultures were incubated in an incubator containing 5% CO₂ in air, other conditions being held the same as reported above.

The CFU-E colonies consisting of 8 or more cells were scored between 36-48 h using an inverted microscope (Leitz, FRG). The BFU-E colonies were scored after 9 days, following the same criteria as for GM-CFC colonies.

5.11 Calculation of total body content of different hemopoietic cells and cell divisions between two cell compartments of a lineage

Total body content was calculated for each stem and progenitor cell compartment by first determining the absolute numbers in total bone marrow. One femur contains 6% of total marrow (Chervenick et al., 1968; Boggs, 1984). The absolute cellularity for a given cell compartment in bone marrow was added to the respective figure in spleen for each mouse individually.

In order to calculate the number of cell divisions, which have occurred in the differentiation and maturation pathway from one relatively immature compartment to a relatively mature compartment, the following formula was used:

No. of cell divisions = $\log$ of the ratio of total body content of two progenitors divided by $\log 2$

For example, the ratio CFU-E : BFU-E is 3.8 and 51 for wild type and mutant mice, respectively. The calculated approximate number of divisions from CFU-E to BFU-E is 2 and 6 for the above genotypes, respectively.

5.12 Preparation of mouse sera and testing for CSF and EPO

Blood was collected (see 5.2) in a disposable plastic tube, allowed to stand at room temperature until it had coagulated, and then kept overnight at 4°C. After centrifugation at 600 g and 4°C for 10 min the serum was withdrawn carefully with a Pasteur pipette and sterilized by filtration through a 0.45 µm millipore filter. The serum was freeze-dried at

-20°C until use.

Test sera of wild type and homozygous mutant mice were diluted serially and prepipetted instead of standard stimulus. The activity of CSF in the sera to be tested was compared with a control of standard MLCM in semisolid agar culture system (see 5.10.1) using BMC of C57Bl/6. To test for EPO present in sera against the standard step III EPO, the methyl cellulose culture system (see 5.10.2) was used. Since the CFU-E of LDH mutants possess a higher plating efficiency and also a slightly greater sensitivity to EPO, BMC of them were used as target cells.

5.13 Proliferation kinetic studies using autoradiography

DNA synthesis time and labeling indices were determined for the different morphologically recognizable erythroblast compartments in wild type and homozygous mutant mice. The method of quantitative ^{14}C -autoradiography (Dörmer, 1973; Dörmer, et al., 1975; Dörmer, 1981) was applied to determine the duration of DNA synthesis. ^{14}C -thymidine (TdR) was used for this purpose instead of ^3H -TdR, since a high degree of β -self-absorption occurs in cells labeled with the latter. Dörmer and Brinkman (1972) found with ^{14}C a maximum autoradiographic loss of 5% radioactivity. ^3H -TdR was used for the labeling index determination because of its well localized blackening in autoradiographs.

5.13.1 Technique of autoradiography

5.13.1.1 Incubation procedure

Spleen cell suspensions of wild type and homozygous mutant mice were prepared separately in TC medium 199 with Hank's salts (Seromed), supplemented with 5×10^{-6} M fluorodeoxyuridine (FUdR) and 10^{-5} M deoxycytidine (CdR). Spleens of two mice of each genotype were processed simultaneously.

As described by Brinkmann and Dörmer (1972), FUdR was used to block the metabolic pathway of endogenous synthesis of thymidine monophosphate (TMP), so that only exogenous labeled TdR (added afterwards) could be converted into TMP. Exogenous CdR was added to overcome the inhibitory effect of thymidine triphosphate on the endogenous formation of CdR.

The cell suspensions were filtered separately through a glass pore filter with a pore width of 45-90 μm (No. D2, Schott, FRG) in order to acquire an absolute single cell suspension. A slight vacuum generated by means of a water jet pump was applied. The cell concentration in each suspension was determined. According to Dörmer and Brinkmann (1970) this was kept below 20,000/ μl to prevent a decrease in the rate of TdR incorporation. 0.95 ml of each cell suspension was pipetted into each of the three plastic tubes, marked 1c, 1a, 1d; the next three were marked 2c, 2a, 2d. Incubation was performed in a water shaking bath at a shaking frequency of approximately 100 strokes/min and at a mean temperature of 37.2°C ($\pm 0.2^\circ\text{C}$). Each tube with cell suspension was treated according to the schedule below:

Tube	Compound	Placed in water	Compound added	Placed in ice
		bath (min)	(min)	(min)
1c	^3H -TdR	1	1	16
2c	^3H -TdR	2	2	17
1a	^{14}C -TdR	3	9	16
2a	^{14}C -TdR	4	10	17
1d	^{14}C -TdR	5	11	14
2d	^{14}C -TdR	6	12	15

The specific activities and the molar concentrations of the stock solutions of the labeled compounds were as follows: for ^3H -TdR 74 GBq/mmol (2 Ci/mmol) and 2×10^{-5} M; for ^{14}C -TdR 2.183 GBq/mmol (59 mCi/mmol) and 2×10^{-4} M. When 0.05 ml of each of these stock solutions were added to 0.95 ml of cell suspension the final concentrations of the labeled compounds were 10^{-6} M and 10^{-5} M, respectively.

The above procedure of incubation was based on the biochemical findings of Brinkmann and Dörmer (1972). After a period of 6 min preincubation with FUdR, which facilitates the disappearance of endogenous TMP, the rate of incorporation of labeled TdR reaches its maximum. Moreover, the rate of TdR incorporation (after 6 min preincubation with FUdR) into DNA is linear during the period between 3rd and 7th min of incubation with TdR. TdR-incorporation could be stopped abruptly by placing the tubes directly into ice. All the cell suspensions were centrifuged at 700 g for 10 min at -4°C . The supernatants were discarded. A drop of serum was added to each pellet, mixed gently and from each sample 10 smears were drawn in a serpentine fashion. For quantitative autoradiography half of a slide was left free to attach the radioactive reference source afterwards. The preparations were air-dried and

fixed in absolute methanol for 48 h with one change after 24 h (Dörmer, a laboratory manual).

5.13.1.2 Autoradiography

A ^{14}C -standard source made of polymethyl- ^{14}C -methacrylate was attached to the lower half of each a and d slides (Dörmer, 1973; Dörmer and Brinkmann, 1972). The standard is a $5 \times 5 \times 1 \text{ mm}^3$ plate with a specific activity of 56.98 KBq/ccm ($1.54 \mu\text{Ci/ccm}$) (Amersham, Braunschweig, FRG).

All smears were covered with Kodak AR-10 stripping film. During the exposure traces of metallic silver (latent image) are formed from silver bromide through the β -particles emitted by radioactive sources. The exposition time for ^3H -smears was 5 days, and for the ^{14}C -smears 23 (a slides) and 51 (d slides) days. Finally the slides were developed, by which process silver grains are produced in place of latent images (Dörmer, 1973). The standard plates were removed carefully. The slides were stained for 40 min in a 5% Giemsa (Merck) solution in buffer (Puffer nach Weise, pH 7.2) and air-dried for 24 h. The slides were differentiated with a cold (at maximum 15°C) mixture of 35% ethanol and 65% Puffer nach Weise. By this procedure the excess stain in the film was removed under microscopic control and the cells could retain an optimum color appropriate for the photometric measurement (Dörmer and Brinkmann, 1972).

5.13.2. Evaluation of the preparations

The morphologically recognizable proliferative erythroblast compartments were considered. At least 50 proerythroblasts, as well as 100

basophilic and polychromatic erythroblasts, were evaluated for each parameter and per mouse. Since the orthochromatic erythroblasts do not proliferate any further, they were not considered for the present study.

5.13.2.1 Determination of labeling index

The labeling index of a definite cell compartment is the fraction of DNA synthesizing cells incorporating ^3H -TdR in a defined period of time (here 15 min), expressed as percentage of the total number of cells of that compartment. Labeling indices of all the 3 erythroblast compartments in wild type and homozygous mutant mice were determined.

5.13.2.2 Quantitative measurement of the ^{14}C -TDR label to determine the DNA-synthesis time: photometry of silver grains

During 3 to 7 minutes of incubation the rate of ^{14}C -TdR incorporation remains constant (Brinkmann and Dörmer, 1972). The rate of incorporation of exogenous ^{14}C -TdR, in the absence of endogenous TdR formation (see 5.13.1.1), corresponds to the rate of synthesis of DNA. The duration of S-phase (DNA-synthesis time) can be calculated from the rate of DNA synthesis and the amount of thymine contained in a diploid cell, which amounts to 5230×10^{-18} mols in mouse (Wagner and Dörmer, 1980). In the calculation of the rate of DNA synthesis the harmonic mean should be applied throughout.

The silver grain density over each cell was measured with the help of an incident light microphotometer MPV 2 (Leitz). The underlying photometric principle has been dealt with in detail by Dörmer (1972,

1974). The cells were selected randomly. The vertical illumination of a mercury vapour lamp reflected from the silver grains, i.e., the reflected luminous flux, was processed by a photomultiplier. The photometric value was converted to digital value by a computer (PDP 8e), and the number of silver grain and ^{14}TdR content per cell were stored (Dörmer et al., 1975, 1978). To avoid too high of a background due to several dye components a filter of 436 nm was used. The measuring aperture used for all the cells was 6.8 μm . A background value for each type of cell was subtracted from the silver grain readings. This was determined by measuring an unlabeled cell.

The silver grain density of the cells was compared with that of the standard sources of known radioactivity (56.98 KBq/ccm) in each autoradiograph, and in this way the radioactivity per cell could be computed. From this radioactivity per cell and the specific activity (2.183 GBq/mmol) of the administered $^{14}\text{C-TdR}$, the amount of TdR incorporated during incubation could be calculated (Dörmer, 1973).

All the data stored on a tape were processed further for statistical analysis and biparametric plotting using programs written in FORTRAN IV.

5.14 Determination of cell cycle status of stem and progenitor cells

The experiments to determine the cell cycle status of the hemopoietic cells were carried out following a method of Becker et al. (1965), modified in our laboratory (Mergenthaler et al., 1982). 10^7 BMC or SPC in a volume of 0.8 ml RPMI 1640 were exposed either to 7.4 MBq (200 μCi in 0.2 ml) of tritiated thymidine ($^3\text{H-TdR}$ of high specific activity, 881-

973 GBq/mmol (23.8-26.3 μ Ci/mmol), Amersham Buchler), or to equimolar concentrations of cold TdR. The medium was supplemented with 1% L-glutamine (Gibco), 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Seromed) and 100 units/ml penicillin-streptomycin (Gibco).

Incubation was performed in a shaking water bath (approx. 100 strokes/min) for 20 min at 37.2°C ($\pm$ 0.2°C). 10 ml of ice-cold RPMI 1640 supplemented with 100 μ g TdR/ml and 10% FCS were added to stop the reaction. The tubes were centrifuged at 700 g for 10 min at 4°C. The cells in the pellet were washed two times in the same way, and then resuspended in a small volume appropriate for individual cell cultures. Cells were assayed for CFU-S (see 5.9), GM-CFC (see 5.10.1), BFU-E and CFU-E (see 5.10.2).

5.15 Radiosensitivity

5.15.1 Irradiation

All irradiations were performed with a caesium-137 source (Gammacell 40, Atomic Energy of Canada Ltd., Commercial Products, Ottawa, Canada). The rate was between 1.15 and 1.08 Gy/min over the period of time when the present experiments were done. If not mentioned otherwise, in the CFU-S assays and bone marrow transplantation experiments recipients were irradiated with 8.0 Gy (lethal dose), at which dose an average of less than 0.2 endogenous CFU-S per mouse survived.

5.15.2 Determination of D_{37} value and extrapolation number

As observed in vitro and in vivo the biological activity of most systems declines exponentially when exposed to an increasing dose of radiation or toxic substances. D_{37} is a numerical value in the exponential part of the curve and is the dose at which the surviving fraction is reduced to e^{-1} or 0.37 (Bond et al., 1965). The value on the ordinate at which the ordinate is intersected by the extrapolated exponential part of the curve is called the extrapolation number (Bond et al., 1965).

In the present study the D_{37} values for the different hemopoietic cells were determined. Bone marrow cells of wild type and homozygous mutant mice were irradiated in vitro with 5 different doses of approximately 0.5, 1.0, 1.5, 2.0 and 2.5 Gy and assayed according to the methods described in 5.9 and 5.10. In each assay one aliquot of non-irradiated BMC was considered as control. The number of stem cells and progenitors estimated in the control suspension was the measurement for 100% survival.

5.15.3 Lethal dose_{50/30} ($LD_{50/30}$)

$LD_{50/30}$ is the dose of irradiation causing death of 50% of animals within 30 days (Bond et al., 1965). In the present experiment five groups of wild type and mutant mice were taken, each group consisting of 16 mice.

5.16 Bone marrow transplantation

BMC in a volume of 0.5 ml/mouse were injected into a lateral tail vein of lethally irradiated wild type mice, and lethally or sublethally irradiated mutant mice.

5.17 Blood transfusion

Larger quantities of wild type blood were collected and concentrated. In order to acquire a greater concentration of red blood cells the blood was mixed (1:8) with MTPBS and centrifuged at 210 g for 10 min at room temperature. The supernatant was removed as carefully as possible, the RBC were resuspended in the remaining fluid and counted (see 5.2). More than 5.5×10^9 RBC were injected into a lateral tail vein of each of the mutant mice.

5.18 Technique of splenectomy

The technique of splenectomy was adapted from the procedures described by Cunliffe-Beamer (1983). Mice were anesthetized by injecting intraperitoneally Evipan-Natrium (sodium salt of methyl-cyclohexenyl-methyl-barbituric acid, Bayer, FRG; solution made by dissolving 0.5 mg powder in 30 ml of distilled water) in a dose corresponding to a volume of less than 1% of the body weight, e.g., if the weight of a mouse was 20 g 0.18 ml were injected. After the mice were fully anesthetized, the hairs in an area of approximately 1 cm diameter on the left side of the

abdomen above the spleen were cut very carefully with curved rounded scissors.

On the left side of each mouse a dorsoventral incision (approx. 1 cm) was made in the skin and in the abdominal muscles approximately 0.5 cm below the last rib, parallel to the segmental vessels. The splenic blood vessels were ligated with absorbable surgical thread, Catgut plain 3-0 (Serag-Wiessner Catgutfabriken, Naila, FRG), and then disconnected with a pair of fine scissors. The pedicle was placed in the abdominal cavity. Abdominal muscle and skin were sutured separately with several interrupted sutures with the help of surgical needles E 320/6 (Acufirm, FRG) and a needle-holding forceps. Catgut was used for abdominal muscle, and non-absorbing thread Mersilene green 4-0 (Ethicon, Nodenstadt, FRG) was used for the skin. Lastly liquid spray plaster (Nobecutan spray, Astra Chemicals, Wedel/Holstein, FRG) was sprayed at the joint of the skin. The mice were kept approximately 50 cm below an infra-red light source to avoid any impairment due to cold after the operation, until they were taken to the animal quarters.

5.19 Histological investigations

Most of the histological investigations were carried out in the laboratory of Dr. A. Luz, Institut für Pathologie, GSF, and some in the laboratory of Prof. Dr. R. Burkhardt, Arbeitsgruppe Hämatomorphologie, GSF. The samples of different organs were fixed, embedded either in paraffin or in acrylate, and stained appropriately as needed.

CHAPTER 6. RESULTS AND DISCUSSIONS

6.1 Morphological investigations and some baseline data

6.1.1 Peripheral blood

The peripheral blood values for wild type, heterozygous mutant and homozygous mutant mice are shown in Table 3. The homozygous mutants had a red blood cell (RBC) count half that of the wild type. On the other hand, RBC count as well as all other values in the heterozygous mutant showed no difference from those of the wild type. The homozygotes showed an extremely high (95.3%) reticulocyte count. The level of their hematocrit was significantly decreased, but not to the same degree as the number of RBC, due to an increased mean cellular volume (MCV) of the RBC. The volumes of both reticulocytes and mature erythrocytes contributed to this elevated MCV (Table 3). The diameters of both cell types were significantly larger in homozygous mutants than in the wild type mice (Table 3). The hemoglobin content was significantly lower in homozygous mutants than in the wild type; however, the mean cellular hemoglobin (MCH) was not diminished, since the MCV was larger in homozygous mutants. The mean cellular hemoglobin concentration (MCHC) was significantly lower in the homozygotes. A few nucleated RBC (Table 4) and basophilic stippling were observed in blood films stained with Giemsa.

The number of white blood cells (WBC) did not differ among the three genotypes (Table 3), but some differences between the wild type and mutant mice were observed in the differential counts (Table 4). Neutrophilic granulocytes were significantly increased and the lymphocytes

Table 3. Peripheral blood values in wild type (+/+), heterozygous mutant (+/*) and homozygous mutant (*/*) mice^a

Parameters	+/+	+/*	+/*
Red blood cells ($\times 10^{-6}/\mu\text{l}$)	9.58 $\pm$ 0.49	9.60 $\pm$ 0.58	4.75 $\pm$ 0.93b
Hematocrit (%)	45.5 $\pm$ 2.0	45.3 $\pm$ 1.7	32.5 $\pm$ 7.6b
Mean cellular volume (μm^3)	47.7 $\pm$ 1.8	47.3 $\pm$ 3.0	69.4 $\pm$ 14.8b
Hemoglobin (g/dl)	15.3 $\pm$ 0.9	15.1 $\pm$ 1.2	8.0 $\pm$ 1.9b
Mean cell hemoglobin (pg)	16.0 $\pm$ 1.1	15.8 $\pm$ 1.4	17.2 $\pm$ 3.6
Mean cellular hemoglobin concentration (g/dl)	33.5 $\pm$ 2.1	33.5 $\pm$ 3.2	25.7 $\pm$ 7.0c
Reticulocytes (%)	2.3 $\pm$ 0.4	2.4 $\pm$ 0.4	95.3 $\pm$ 1.2b
Reticulocytes ($\times 10^{-5}/\mu\text{l}$)	2.2 $\pm$ 0.4	2.0 $\pm$ 0.4	46.2 $\pm$ 6.6b
Diameter of erythrocytes (μm)	6.0 $\pm$ 0.6	n. d. ^d	6.7 $\pm$ 0.7b
Diameter of reticulocytes (μm)	6.5 $\pm$ 0.7	n. d. ^d	6.9 $\pm$ 0.7b
White blood cells ($\times 10^{-3}/\mu\text{l}$)	5.0 $\pm$ 2.1	4.9 $\pm$ 1.9	5.0 $\pm$ 1.8

^aData are expressed as mean $\pm$ SD of 12 mice of both sexes. The diameters of 200 cells of each type from 4 mice were examined.

b & c Values significantly different from wild type values according to Student's t-test; $P < 0.0001$ and $P < 0.002$ for b and c, respectively.

^dn. d. = not determined

Table 4. Differential count of nucleated cells in peripheral blood of wild type (+/+), heterozygous mutant (+/++) and homozygous mutant (++) mice^a

Parameters	+/+		+ / ++		++ / ++	
	%	Abs. no./ μ l	%	Abs. no./ μ l	%	Abs. no./ μ l
Metamyelocytes ^b	1.8 $\pm$ 0.3	90 $\pm$ 35	2.2 $\pm$ 0.8	104 $\pm$ 61	1.2 $\pm$ 0.6	55 $\pm$ 31 ^d
Band ^b	3.7 $\pm$ 1.7	183 $\pm$ 98	2.8 $\pm$ 1.1	133 $\pm$ 68	4.0 $\pm$ 1.8	204 $\pm$ 115
Segmented ^b	6.9 $\pm$ 1.9	330 $\pm$ 117	11.5 $\pm$ 4.2	540 $\pm$ 260 ^d	17.1 $\pm$ 7.0	904 $\pm$ 610 ^d
Eosinophil ^c			0.1 $\pm$ 0.2	4.4 $\pm$ 7.2	n. d. ^g	
Total	12.4 $\pm$ 3.1	600 $\pm$ 225	16.5 $\pm$ 5.6	780 $\pm$ 370	22.2 $\pm$ 8.6	1160 $\pm$ 710 ^d
granulocytic lymphocytes	86.7 $\pm$ 2.9	4450 $\pm$ 2060	81.9 $\pm$ 5.4	3840 $\pm$ 1340	76.7 $\pm$ 8.9	3870 $\pm$ 1310
G:L ratio	1:7.5 $\pm$ 2.1		1:5.5 $\pm$ 1.9 ^d		1:4.0 $\pm$ 1.7 ^d	
Monocytes	1.0 $\pm$ 0.5	55 $\pm$ 52	1.6 $\pm$ 0.5	78 $\pm$ 37	0.7 $\pm$ 0.6	32 $\pm$ 26
Polychromatic erythroblasts	---	---	---	---	0.4 $\pm$ 0.4	20 $\pm$ 25
Orthochromatic erythroblasts	---	---	---	---	0.1 $\pm$ 0.2	5.1 $\pm$ 8.5
Total erythroid	---	---	---	---	1.2 $\pm$ 0.9	25 $\pm$ 24

^aData are expressed as mean $\pm$ SD of 10 mice of both sexes; at least 250 cells per mouse were counted

^bNeutrophilic cell type

^cEosinophilic cells were present only in very low numbers in all the genotypes, however, not observed in +/+ and +/++ mice during differential count.

^dValues significantly different from wild type values according to Student's t-test (only absolute values were considered for this test).

es. s. = Statistical significance f n. s. = not significant gn. d. = not determined

slightly decreased in the homozygous mutants. Metamyelocytes, and especially segmented granulocytes, contributed to that increase. The G:L ratio of the homozygous mutants was significantly different (at the level of 0.1%) from that of the wild type mice. In heterozygotes, only the number of segmented granulocytes was found to be different from that of wild type mice at the 5% level of significance. Although the total granulocytic count of the former was not significantly different from the wild type value, a slight decrease in lymphocyte count resulted in a significantly (at the level of 5%) different G:L ratio.

6.1.2 Bone marrow

The total number of bone marrow cells (BMC) in femurs of the mutants did not differ significantly from the wild type value (Table 7). Table 5 shows the differential counts of BMC in femurs of wild type and homozygous mutant mice. Although the number of promyelocytes and myelocytes was lower in the homozygous mutants than in the wild type, the number of total granuloid cells was similar in both genotypes. The absolute number of lymphocytes was slightly higher in the mutants, but again, the total number of lymphoid cells showed no genotypic difference. On the contrary, except for proerythroblasts all erythroid cell types as well as the G:E ratio were significantly (at the level of 0.01%) higher in the homozygous mutants.

The histological investigations of the vertebral bone marrow revealed a more compact cellularity in the homozygous mutants than in the wild type mice (Figs. 2a and 2b). Preparations specially stained for hemosiderin detection showed that the mutants had less hemosiderin deposition than the wild type. The mutant bone marrow showed a compara-

Table 5. Differential count of bone marrow cells in femora on wild type (+/+) and homozygous mutant (+*/+*) mice^a

Parameters	+/+		+*/+*		Statistical significance
	%	Total number ^c	%	Total number ^c	
Myeloblasts	0.6 ± 0.4	75 ± 58	0.9 ± 0.5	141 ± 86	n. s. f
Promyelocytes ^b	2.8 ± 0.9	365 ± 151	1.2 ± 0.6	202 ± 96	p<0.01
Myelocytes ^b	18.6 ± 3.6	2350 ± 600	10.7 ± 3.0	1760 ± 640	p<0.05
Metamyelocytes ^b	4.0 ± 1.5	500 ± 186	4.0 ± 1.3	640 ± 210	n. s.
Band ^b	16.3 ± 5.5	2030 ± 710	16.7 ± 4.2	2720 ± 840	n. s.
Segmented ^b	6.4 ± 2.6	840 ± 468	4.2 ± 1.4	680 ± 204	n. s.
Eosinophils	2.0 ± 0.7	256 ± 103	1.4 ± 0.9	228 ± 136	n. s.
Total granulocytic	50.8 ± 6.3	6420 ± 1480	39.1 ± 6.6	6370 ± 1530	n. s.
Lymphoblasts	0.7 ± 0.3	84 ± 48	0.5 ± 0.3	83 ± 20	n. s.
Lymphocytes	29.0 ± 4.0	3700 ± 940	27.8 ± 3.6	4490 ± 730	p<0.05
Total lymphoid	29.6 ± 4.2	3780 ± 980	28.2 ± 3.4	4570 ± 750	n. s.
G:L ratio	1 : 0.60 ± 0.14		1 : 0.75 ± 0.20		n. s.
Proerythroblasts	0.4 ± 0.2	43 ± 26	0.5 ± 0.3	75 ± 55	n. s.
Basophilic EB ^e	1.1 ± 0.7	136 ± 102	2.3 ± 1.0	379 ± 155	p<0.0001
Polychromatic EB	9.8 ± 2.9	1240 ± 446	17.1 ± 3.7	2820 ± 860	p<0.0001
Orthochromatic EB	7.7 ± 2.6	950 ± 316	12.2 ± 3.8	1940 ± 550	p<0.0001
Total erythroid	18.9 ± 5.1	2380 ± 770	32.1 ± 4.6	5210 ± 1000	p<0.0001
G:E ratio	1 : 0.4 ± 0.1		1 : 0.9 ± 0.3		p<0.0001
Monocytes	0.3 ± 0.1	39 ± 18	0.5 ± 0.2	69 ± 26	n. d. g
Plasma cells	0.2 ± 0.2	26 ± 31	0.1 ± 0.1	9 ± 15	n. d.
Megakaryocytes	0.1 ± 0.1	11 ± 14	0.1 ± 0.1	28 ± 35	n. d.

^aData are expressed as mean ± SD of 10 mice of both sexes; at least 500 cells per mouse were counted.

^bNeutrophilic cell types. ^cTotal number x 10⁻³/femur

^dThe Student's t-test was applied and only absolute values were compared

^eEB = erythroblasts ^fn. s. = not significant ^gn. d. = not determined

Table 6. Differential count of spleen cells in wild type (+/+) and homozygous mutant (+*/+*) mice^a

Parameters	+/+		+*/+*		Statistical significance
	%	Total number ^c	%	Total number ^c	
Myeloblasts	0.6 ± 0.3	5.0 ± 3.7	0.6 ± 0.3	104 ± 65	n. s. f
Promyelocytes ^b	1.2 ± 0.5	9.7 ± 4.6	1.2 ± 0.4	200 ± 174	n. s.
Myelocytes ^b	4.7 ± 1.7	37.8 ± 18.6	6.5 ± 1.5	1160 ± 720	p<0.05
Metamyelocytes ^b	1.2 ± 0.4	9.2 ± 3.2	0.9 ± 0.6	152 ± 136	n. s.
Band ^b	1.6 ± 0.9	12.1 ± 7.2	0.8 ± 0.8	133 ± 127	n. s.
Segmented ^b	2.6 ± 1.9	19.6 ± 14.0	0.9 ± 0.6	130 ± 77	p<0.02
Eosinophils	0.4 ± 0.3	3.0 ± 1.9	0.2 ± 0.2	30 ± 37	n. s.
Total granulocytic	12.4 ± 3.5	96.4 ± 37.1	10.8 ± 3.2	1910 ± 1160	n. s.
Lymphoblasts	0.8 ± 0.5	6.4 ± 4.0	0.6 ± 0.3	108 ± 89	n. s.
Lymphocytes	76.9 ± 4.6	600 ± 115	22.8 ± 6.8	3910 ± 1770	p<0.0001
Total lymphoid	77.7 ± 4.7	600 ± 115	23.4 ± 6.7	4020 ± 1830	p<0.0001
G:L ratio	1 : 6.8 ± 2.1		1 : 2.3 ± 1.0		p<0.0001
Proerythroblasts	0.5 ± 0.3	4.1 ± 2.8	1.2 ± 0.7	193 ± 90	p<0.02
Basophilic EBe	1.3 ± 0.5	9.3 ± 3.9	13.1 ± 5.1	2510 ± 1800	p<0.0001
Polychromatic EB	3.4 ± 1.9	33.4 ± 18.7	33.3 ± 5.4	5700 ± 2540	p<0.0001
Orthochromatic EB	3.0 ± 1.0	24.0 ± 10.5	18.0 ± 3.9	3070 ± 1500	p<0.0001
Total erythroid	9.1 ± 2.8	71.0 ± 30.8	65.5 ± 7.8	9480 ± 4250	p<0.0001
G:E ratio	1 : 0.8 ± 0.3		1 : 6.7 ± 2.3		p<0.0001
Monocytes	0.1 ± 0.1	1.0 ± 1.0	0.1 ± 0.1	20 ± 22	n. d. g
Plasma cells	0.9 ± 0.6	7.0 ± 4.8	0.1 ± 0.1	11 ± 15	n. d.
Megakaryocytes	0.04 ± 0.08	0.3 ± 0.6	0.1 ± 0.1	20 ± 22	n. d.

^aData are expressed as mean ± SD of 10 mice of both sexes; at least 500 cells per mouse were counted

^bNeutrophilic cell types

^cTotal number x10⁻⁵/spleen

^dThe Student's t-test was applied and only relative values were compared

eEB = erythroblasts f n. s. = not significant gn. d. = not determined

tively immature picture with more histiophagocytes containing nuclear rests (especially normoblasts) than the wild type bone marrow.

6.1.3 Spleen

The spleen weight was about 7 times higher and the splenic cellularity 14 times higher in the homozygous mutants than in the wild type (Table 7). Spleen weight in heterozygous mutants was higher than in the wild type, although not at a level of significance.

The differential counts of spleen cells (SPC) in the wild type and homozygous mutant mice are shown in Table 6. The mutants showed a low percentage of lymphoid cells which was highly significant. The percentage of granuloid cells was almost the same in both genotypes. The mutants showed an average G:L ratio of 1:2.3, significantly different from that of wild type mice (1:6.8). The fraction of erythroid cells was more than 65% in mutants, while it was only 9% in the wild type. In the mutants, the granulocytic, lymphoid and erythroid populations were elevated several times in the spleen. As compared to those of the wild type, the expansion of the erythroid cells in the mutants (134 times) were much higher than those of the other two (20 and 7 times, respectively).

The histopathological investigations of the homozygous mutant spleens showed a large number of erythropoietic foci and hemosiderin positive regions, whereas the wild type mice showed only rare erythropoietic foci in the spleen and no siderosis.

Table 7. Cellularities, spleen weights and LDH activities for wild type (+/+), heterozygous mutant (+/+) and homozygous mutant (++) mice^a

Parameters	+/+	+/+	++/++
BMC ($\times 10^{-6}$ /femur)	14.0 $\pm$ 4.1 (46)	13.4 $\pm$ 2.7 (35)	15.6 $\pm$ 4.0 (53)
SPC ($\times 10^{-6}$ /spleen)	110 $\pm$ 62 (30)	196 $\pm$ 128 (26)	1650 $\pm$ 623 (26)
Spleen weight (mg.)	107 $\pm$ 38 (66)	123 $\pm$ 43 (76)	758 $\pm$ 131 (53)
LDH activities			
In blood (U/ 10^9 RBC)	4.03 $\pm$ 0.82 (24)	2.18 $\pm$ 0.26 (12)	0.36 $\pm$ 0.18 (25)
In BMC (U/ 10^9 cells)	13.90 $\pm$ 2.19 ^b	n. d. ^c	1.07 $\pm$ 0.44 ^b

^aData are expressed as mean $\pm$ SD of mice of both sexes; figures in parenthesis represent numbers of animals examined.

^bMean $\pm$ SD of 3 measurements; each cell suspension was pooled from 8 femurs and 8 tibias.

^cn. d. = not determined

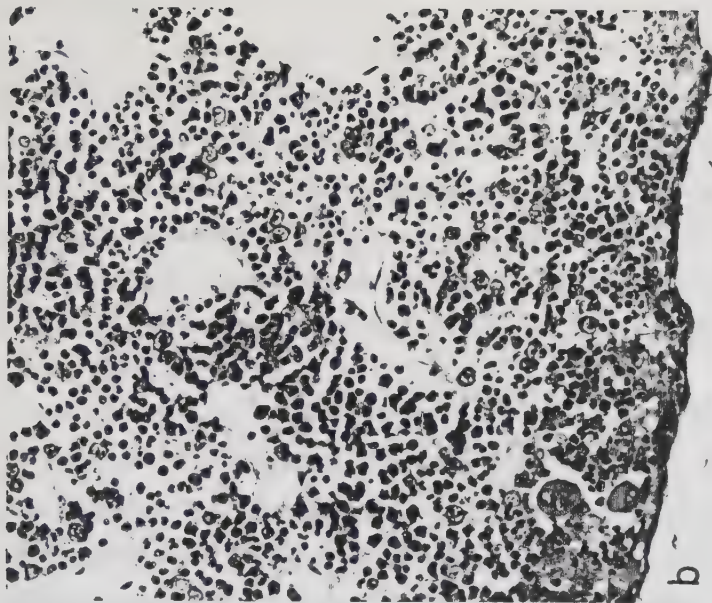
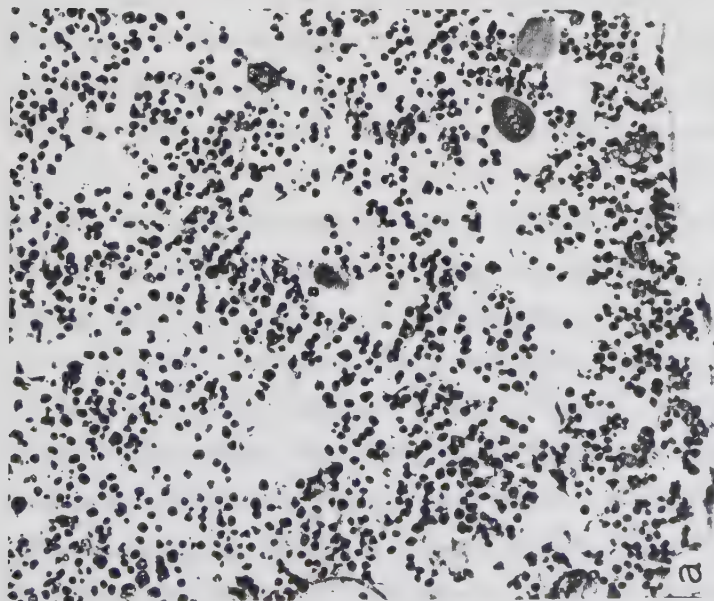


Fig. 2. Vertebral marrow of wild type (a) and homozygous mutant (b) mouse showing uniform distribution of cells and difference in compactness of the cells between the genotypes. Cross sections 3 μ m; stain galamin blue-Giemsa; 250 x ((original)).

6.1.4 LDH activities

LDH activities were determined by Pretsch and Charles (1987) in different organs (Table 1). In the present project LDH activities were determined in peripheral blood and BMC (Table 7). LDH activities in peripheral blood of heterozygous and homozygous mutants were about 50% and 9%, respectively, of the wild type values. In the BMC of homozygous mutants, LDH activities were about 8% of that of the wild type.

6.1.5 Discussion

In the homozygous mutants, the low number of RBC, the extremely high reticulocytosis and a splenomegaly are the clinical manifestations of hemolytic anemia. Several inherited hemolytic anemias in mice have been described (Bernstein, 1980; Hutton and Bernstein, 1973; Russel, 1979; Unger et al., 1983). In these cases the hemolytic disease is caused by a deficiency of the red cell membrane protein spectrin, without any defect in the glycolytic pathway (Lux et al., 1981; Unger et al., 1983).

The erythrocyte life span in C3H mouse has been estimated to be 40 to 43 days (von Ehrenstein, 1958). In the homozygous mutant, the RBC count diminishes very rapidly after irradiation with 5 or 8 Gy (see 6.7.4). Following irradiation with 5.0 Gy, the $t_{1/2}$ of the RBC was calculated to be 1.6 days. Moreover, the high percentage of reticulocytes, and the presence of polychromatic and orthochromatic erythroblasts emphasize the fact that mutants have an extremely high rate of RBC production. The diminished amount of MCHC in homozygous mutants may be due to the presence of the large number of reticulocytes with incom-

plete hemoglobinization.

The cause of the hemolytic disease in the mutants is presumed to be an effect of the LDH mutation. It is supposed that the erythrocytes of the mutants with about 9% LDH activity of the wild type can function only for a few days. The actual biochemical events between enzyme deficiency and destruction of the erythrocytes are at present unknown. The erythrocytes of the heterozygous mutants, with about 50% LDH activity of the wild type, seem to produce enough energy to maintain normal steady state conditions.

The numerous hemolytic anemias in man caused by different inherited enzyme deficiencies have been reviewed by Wintrobe (1981) and Valentine (1984). Unlike the mouse, the erythrocytes in man contain predominantly LDH 2 (H₃M) and LDH 1(H₄) (Jungermann and Möhler, 1980). Up to now only one patient has been reported with an LDH deficiency (Kitamura et al., 1971; Miwa et al., 1971). A 64 year-old Japanese male had a hereditary deficiency of the H-subunit of LDH and only possessed LDH composed of the M-subunit. The LDH activity in the erythrocytes was very low (5.7% of normal), but other blood parameters suggested no clinical manifestations of hemolytic anemia. The inherited deficiency was autosomal and codominant like that of the present LDH mutant mouse.

One possible reason of the low hemosiderin in the bone marrow of mutants may be that it is stored in other organs, such as the spleen, liver and kidneys. In fact, in the homozygous mutants, several Kupffer cells in the liver and the distal cortex tubules in the kidney have been observed with siderosis. Mice made anemic by feeding with toluidine blue also showed an excess of hemosiderin in the kidney (Dunn, 1967).

6.2 Quantification and calculation of total body content of different hemopoietic cells

6.2.1 Frequencies and numbers of colony-forming units-spleen (CFU-S), granulocyte-macrophage colony-forming cells (GM-CFC), burst-forming units-erythroid (BFU-E), and colony-forming units-erythroid (CFU-E)

The frequencies and numbers of different hemopoietic cells assayed in wild type (+/+), heterozygous mutant (+/+*) and homozygous mutant (+*/+*) mice are shown in Table 8. The pluripotent hemopoietic stem cells (CFU-S) were determined by CFU-S assay (see 5.9). The number of CFU-S per femur was slightly lower in the +/+* and +*/+* than in the +/+ mice. On the other hand, CFU-S content per spleen of +*/+* mice was about 9 times higher than in the +/+ mice. The CFU-S colony counts were not corrected for seeding efficiency, since the values did not differ much from each other. Seeding factor f (see 5.9) for bone marrow-derived CFU-S in the +/+ and +*/+* mice was 0.026 ± 0.004 and 0.023 ± 0.005 (mean $\pm$ SEM), respectively (Table 9). Seeding factor was not determined in heterozygous mutants and it was presumed that the factor would be comparable to that of the wild type, since the results obtained in the two genotypes were very similar.

Granulocyte-macrophage and erythroid progenitors were investigated by semisolid culture techniques (see 5.10). The frequencies and numbers of all progenitors in heterozygous mutants were very much comparable to those of the wild type. In homozygous mutants the femur GM-CFC values were significantly lower than in wild type. The BFU-E values in femur were similar in both of these genotypes. However, in the spleen of

Table 8. Hemopoietic stem cells and progenitors in bone marrow and spleen of wild type (+/+), heterozygous mutant (+/++) and homozygous mutant (++) mice^a

Cells assayed	+/+	+/++	++/++	Factor	
				+/+	++/++
CFU-Sb	per 10 ⁵ BMCE	15.8 ± 0.5	13.7 ± 2.4	15.2 ± 1.3	0.87
	per femur (x10 ⁻³)	1.80 ± 0.08	1.28 ± 0.34	1.34 ± 0.20 ^f	0.71
	per 5x10 ⁵ SPCe	12.9 ± 3.3	16.9 ± 4.8	12.1 ± 2.8	1.30
	per spleen (x10 ⁻³)	1.60 ± 0.76	2.17 ± 0.76	14.7 ± 1.7 ^f	1.40
GM-CFCC	per 5x10 ⁴ BMC	65.2 ± 6.0	54.4 ± 4.1	38.7 ± 3.8 ^f	0.59
	per femur (x10 ⁻³)	17.1 ± 2.1	12.8 ± 1.5	9.67 ± 1.32 ^f	0.74
	per 10 ⁶ SPC	79.4 ± 14.1	114 ± 27	101 ± 9	1.40
	per spleen (x10 ⁻³)	6.96 ± 1.29	12.6 ± 4.1	157 ± 31 ^f	1.80
BFU-Ed	per 10 ⁵ BMC	28.9 ± 2.2	27.6 ± 2.3	30.0 ± 3.4	1.00
	per femur (x10 ⁻³)	3.86 ± 0.45	3.27 ± 4.84	3.52 ± 0.39	0.85
	per 10 ⁶ SPC	10.2 ± 1.0	13.4 ± 1.3	19.6 ± 1.8	1.3
	per spleen (x10 ⁻³)	1.49 ± 0.36	1.92 ± 5.46	31.8 ± 4.0	21.0
CFU-Ed	per 10 ⁵ BMC	78.2 ± 6.9	76.7 ± 4.4	194 ± 14 ^f	0.98
	per femur (x10 ⁻³)	10.3 ± 1.1	9.23 ± 5.73	23.7 ± 2.2 ^f	0.90
	per 10 ⁵ SPC	52.5 ± 4.4	63.3 ± 5.8	257 ± 15 ^f	1.2
	per spleen (x10 ⁻³)	76.9 ± 19.9	92.8 ± 16.9	4180 ± 365 ^f	1.2
					54.0

^aData are expressed as mean ± SD of individually tested male mice

bCells from 3 mice of each genotype were assayed in 3 separate experiments. Each cell suspension was injected into 6 lethally irradiated recipients. Data are not corrected for seeding efficiency.

cCells from 12 mice of each genotype were assayed in 4 separate experiments. Each cell suspension was tested in triplicate cultures.

dCells from 10 mice of each genotype were assayed in 5 experiments. Each cell suspension was tested in triplicate (for CFU-E) or quadruplicate (for BFU-E) cultures.

eBMC = viable nucleated bone marrow cells SPC = viable nucleated spleen cells

^fSignificantly different from wild type values (p<0.01 by the Wilcoxon test)

Table 9. Seeding factor f for bone marrow-derived CFU-S in wild type (+/+) and homozygous mutant (+*/+*) mice^a

Parameters	+/+	+*/+*
CFU-S per 10 ⁵ BMC ^b	8.8 ± 0.8	7.4 ± 0.9
No. of BMC injected per primary recipient ^c	2 × 10 ⁷	2 × 10 ⁷
Total no. of CFU-S injected per primary recipient	1760 ± 150	1480 ± 180
Total no. of CFU-S recovered ^d	45 ± 8	34 ± 8
Seeding factor f	0.026 ± 0.004	0.023 ± 0.005

^aData are expressed as mean ± SEM. Spleen colonies were counted 12 days after transplantation of BMC.

^bNumber of CFU-S in bone marrow cell suspension was determined by injecting 10⁵ BMC into each of the 10 recipients (see 5.9).

^c4 primary recipients were taken.

^dSpleens of the primary recipients were removed 24 h after transplantation and the equivalent of 1/10 spleen was injected into each of 8 secondary recipients.

+*/+* mice both GM-CFC and BFU-E values were significantly higher (20 times). Femoral CFU-E values in +*/+* mice were more than twofold increased in comparison to that of the wild type, while the splenic CFU-E were more than 50 times higher, since the frequency was markedly elevated (about 5 times).

6.2.2 Total body content of the different hemopoietic cells

Total body content values for hemopoietic stem and progenitor cells were determined in all 3 genotypes (see 5.11). This approach is based on the restriction of hemopoiesis to bone marrow and spleen. In fact,

Table 10. Total body content of hemopoietic stem and progenitor cells in wild type (+/+), heterozygous mutant (+/+*) and homozygous mutant (+*/+*) mice^a

Cells assayed	+/+	+/*	+*/+*	Factor	
				+/*	+/*/+*
CFU-Sb	31.6 ± 0.8	23.4 ± 5.2	37.2 ± 3.7	0.74	1.18
GM-CFCC	277 ± 35	225 ± 23	318 ± 49	0.81	1.15
BFU-Ed	65.8 ± 7.7	55.6 ± 4.9	90.6 ± 8.4 ^e	0.84	1.38
CFU-Ed	248 ± 29	247 ± 18	4580 ± 340 ^f	1.0	18.5

^aData are expressed as mean ± SEM of individually tested male mice.

^bCells from 3 mice of each genotype were assayed in 3 separate experiments. Each cell suspension was injected into 6 lethally irradiated recipients. Data were not corrected for seeding efficiency. No Wilcoxon test was possible, since only 3 total body content values were available.

^cCells from 12 mice of each genotype were assayed in 4 separate experiments. Each cell suspension was tested in triplicate cultures.

^dCells from 10 mice of each genotype were assayed in 5 separate experiments. Each cell suspension was tested in triplicate, for CFU-E, and quadruplicate, for BFU-E, cultures.

^e & ^fSignificantly different from wild type values ($P < 0.002$ and $P < 0.05$, respectively) determined by Wilcoxon test.

histological investigations of various organs (heart, lung, liver, pancreas, thymus, lymph nodes, kidney and testicle) could not detect any trace of erythropoiesis. Table 10 shows the values for total body content. The CFU-S and GM-CFC values were comparable among the 3 genotypes. The total BFU-E pool was slightly higher in the +*/+* mice. The CFU-E pool showed the most remarkable difference being 18 fold higher in the homozygous mutants.

Table 11. Contribution of the spleen to total hemopoiesis in wild type (+/+) and homozygous mutant (+*/+*) mice^a

Cells assayed	Splenic stem or progenitor cells (% of total body content)	
	+/+	+*/+*
CFU-S	5 ± 2	40 ± 5
GM-CFC	3 ± 1	48 ± 3
BFU-E	2 ± 0.3	35 ± 3
CFU-E	29 ± 4	91 ± 2

^aData are expressed as mean ± SEM of individually tested male mice.

Other details are same as in Table 10.

6.2.3 Contribution of spleen to total hemopoiesis

The enlarged spleen of the homozygous mutants was found to contribute an essential part to total hemopoiesis of the mouse. Table 11 shows that the fractions of CFU-S and all the progenitors are much higher in the mutants. Especially noteworthy is the large fraction of CFU-E. The spleen of the homozygous mutant produces more than 90% of the total body CFU-E.

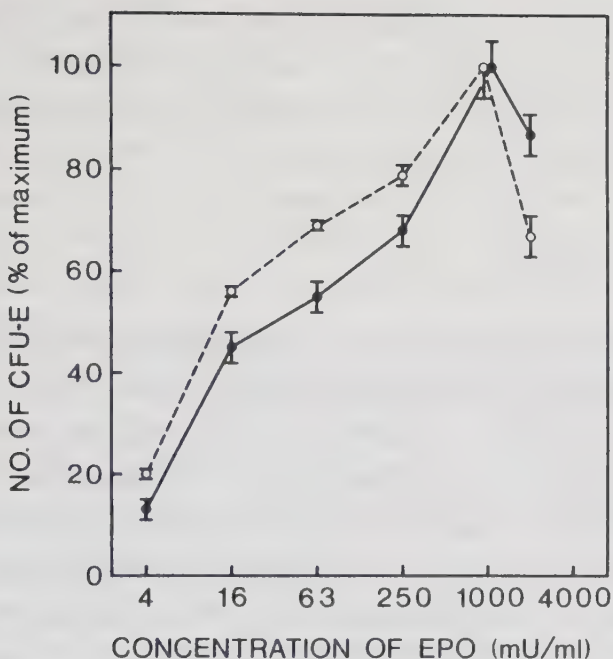


Fig. 3. Responsiveness of CFU-E in the bone marrow of wild type (closed symbols, full lines) and homozygous mutant (open symbols, dashed lines) mice to different concentrations of erythropoietin. Triplicate cultures were performed and colonies were scored between 36-48 h. Each point with vertical bars represents the mean $\pm$ SD obtained from one experiment. Two other experiments showed similar results.

6.2.4 Responsiveness of progenitors to hemopoietic stimulating factors

The responsiveness of bone marrow-derived GM-CFC and BFU-E was similar in the wild type and homozygous mutant mice; the same was observed for the responsiveness of BFU-E to BPA. This was determined in cultures containing serial dilutions of those stimulators in a preliminary experiment (data not shown). CFU-E derived from bone marrow of mutants

and cultured in serial dilutions of EPO showed a slightly higher responsiveness in the lower dilution range than those from the wild type. However, the concentration of EPO needed to obtain maximum stimulation was the same in both genotypes (Fig. 3).

6.2.5 Discussion

Data acquired in the above series of experiments indicate that marrow erythropoiesis in the homozygous mutants is stimulated at the level of CFU-E; CFU-S, GM-CFC and BFU-E are quantitatively lower or the same as in wild type mice. Splenic hemopoiesis, on the other hand, is largely expanded (9-50 times) (Table 8). Heterozygous mutants do not show remarkable differences from the wild type except for a slightly lower frequency of bone marrow-derived CFU-S. Of the various murine hemolytic anemias described (Bernstein, 1980; Lux et al., 1981) no complete set of data is available making possible a meaningful comparison with the present findings. Hemolytic anemia induced in mice or rats by several injections of phenylhydrazine at short intervals showed comparable results. The spleen was enlarged, the stem and progenitor cells within it expanded several times. However, they were more or less diminished in the bone marrow, except for CFU-E which were elevated in the marrow (Rencricca et al., 1970; Hara and Ogawa, 1976; Hodgson et al., 1972).

Total body content calculations were done for stem cells and each type of progenitor cells in order to quantify the contribution of those hemopoietic compartments to the compensatory mechanism in the hemolytic disease of the homozygous mutants (Table 10). The enlarged spleen contained about 9 and 20 fold more CFU-S and GM-CFC, respectively, which was enough to compensate for the deficit in those compartments in

the bone marrow. However, the total pools of CFU-S and GM-CFC in both genotypes were comparable. The total body content of BFU-E in the homozygous mutant mice was slightly higher than in the wild type mice, but the number of CFU-E was very striking (more than 18 times higher) in the mutants. This expansion agreed in magnitude with the number of reticulocytes (Table 3) in the peripheral blood of the mouse, which was 20 times higher than in normal mice. Hodgson (1985) performed a quantitative evaluation of the hemopoietic stem and progenitor cells in animals with phenylhydrazine-induced acute hemolytic anemia, using data of several investigators. He found only minor changes in the total number of CFU-S, while GM-CFC and BFU-E numbers were decreased. The CFU-E numbers were 17 times increased, half of which was accounted for by the spleen. In the present mutant the latter value is more striking, since more than 90% of the elevation was contributed by the spleen. Therefore, it can be concluded that protracted and severe red blood cell loss is compensated for by an expansion of the more mature erythroid precursors. As it has been envisaged by Hodgson (1985) and Rencricca et al. (1970), the cause of the lower number of CFU-S and GM-CFC in the bone marrow and the increased number in the spleen may be attributed to migration of stem cells from bone marrow to spleen in order to fulfill the demands of the expanded spleen.

6.3 Hemopoietic changes following splenectomy in wild type and homozygous mutant mice

The normal mouse spleen is an active hemopoietic organ and significantly contributes to total hemopoiesis (Bozzini et al., 1970; Fruhman, 1970). On the other hand, splenic macrophages are actively engaged in the recognition and subsequent destruction of senescent erythrocytes by a previously detected auto-immune mechanism (Singer et al., 1986). Therefore, the spleen's dual role as a significant production site for new RBC and as a sequestering organ for senescent red cells had to be considered. It is known from other strains of mice with congenital hemolytic anemias that the spleens expand severalfold in order to compensate for the high degree of red cell loss (Bernstein, 1980). The enlarged spleen (7-10 times normal) of the LDH homozygous mutant mice has a significantly greater contribution to total body hemopoiesis than that of the wild type mice (Table 11). The fraction of CFU-E in the mutant spleen is most striking: 91% of the total body CFU-E are found in the spleen, which therefore appears to be an indispensable organ for this specific precursor production. Splenectomy experiments were carried out to see whether the mutants were able to survive at all after splenectomy, or, conversely, whether splenectomy could alleviate the hemolytic disease. In pilot studies it was found that the mutants could indeed survive. In order to investigate this particular hemopoietic situation, long-term effects of splenectomy were analyzed. Wild type and homozygous mutant mice were examined at weeks 5, 9, 13 and 16 following splenectomy. At each time point 3 splenectomized and 3 control mice (non-splenectomized) of each genotype were examined. All control values from peripheral blood have been combined for the calcu-

lation of the mean $\pm$ SD. For determination of stem cell and progenitor population sizes in each splenectomized and control group, pooled femoral bone marrow cells from 3 mice were studied.

6.3.1 Peripheral blood values, LDH activity and other remarkable observations

Table 12 shows the peripheral blood values and LDH activity in splenectomized wild type and homozygous mutant mice. The RBC count in wild type mice was below the normal level up to week 13 and then normalized. On the contrary, in splenectomized mutants it was extremely low, about 1/3 of the RBC count in non-splenectomized mutants. Hematocrit values in the wild type showed almost the same course as the RBC count, since there was no virtual change in the MCV. In the mutants, the hematocrit values were diminished to roughly 50% of untreated animals, resulting in about a 150% increase of the MCV. Erythrocyte hemoglobin content in the wild type mice was only slightly decreased, while in the mutants it was almost half the control value. The percentage as well as the absolute number of reticulocytes was found to be increased in the wild type mice up to week 13 and then normalized. In contrast, in the mutants the fraction declined gradually to about 80% of the total RBC. The absolute value was even more diminished, since the RBC count was very low.

As a consequence of splenectomy anisocytes and poikilocytes were observed in the blood of both mutant and wild type mice. However, these alterations of RBC morphology were more frequent and more pronounced in the mutants. In addition, post-splenectomy Howell-Jolly bodies were observed in both genotypes. A significant increase in the number and size of platelets was observed in the blood films of both groups (data)

Table 12. Peripheral blood values and LDH activities in splenectomized wild type (+/+) and homozygous mutant (+*/+*) mice^a

Parameters	Geno- type	Control values	5	Weeks after 9	splenectomy 13	16
Red blood cells ($\times 10^{-6}/\mu\text{l}$)	+/+	8.7 $\pm$ 0.4	7.5 $\pm$ 0.5	7.7 $\pm$ 0.2	7.7 $\pm$ 0.7	9.6 $\pm$ 1.3
	+*/+*	4.7 $\pm$ 0.5	1.7 $\pm$ 0.2	1.5 $\pm$ 0.2	1.6 $\pm$ 0.2	1.7 $\pm$ 0.03
Hematocrit (%)	+/+	41.9 $\pm$ 2.2	37.1 $\pm$ 1.9	38.5 $\pm$ 1.3	34.3 $\pm$ 3.8	45.1 $\pm$ 1.5
	+*/+*	33.2 $\pm$ 1.6	17.6 $\pm$ 0.6	16.3 $\pm$ 18	15.3 $\pm$ 1.0	16.5 $\pm$ 1.4
Mean cellular volume (μm^3)	+/+	48.2 $\pm$ 2.9	49.6 $\pm$ 1.8	49.8 $\pm$ 1.3	44.8 $\pm$ 0.9	47.6 $\pm$ 4.7
	+*/+*	70.9 $\pm$ 5.8	107 $\pm$ 11.2	111 $\pm$ 6.5	97.9 $\pm$ 9.1	97.6 $\pm$ 6.9
Hemoglobin (g/dl)	+/+	14.2 $\pm$ 1.2	13.8 $\pm$ 0.9	12.4 $\pm$ 0.7	12.3 $\pm$ 1.6	13.8 $\pm$ 3.9
	+*/+*	9.3 $\pm$ 0.8	5.4 $\pm$ 0.7	4.0 $\pm$ 0.4	5.0 $\pm$ 0.7	4.2 $\pm$ 0.4
Reticulocytes (%)	+/+	2.3 $\pm$ 0.5	4.9 $\pm$ 0.4	4.0 $\pm$ 1.2	4.2 $\pm$ 1.1	1.8 $\pm$ 0.3
	+*/+*	95.4 $\pm$ 1.0	92.8 $\pm$ 0.7	89.4 $\pm$ 2.0	89.9 $\pm$ 1.2	80.5 $\pm$ 1.9
Reticulocytes ($\times 10^{-5}/\mu\text{l}$)	+/+	2.0 $\pm$ 0.4	3.7 $\pm$ 0.1	3.1 $\pm$ 0.9	3.2 $\pm$ 0.6	1.7 $\pm$ 0.4
	+*/+*	44.9 $\pm$ 4.6	15.5 $\pm$ 2.0	13.2 $\pm$ 2.3	14.2 $\pm$ 1.6	13.6 $\pm$ 0.5
White blood cells ($10^{-3}/\mu\text{l}$)	+/+	5.3 $\pm$ 0.8	n. d. ^c	8.9 $\pm$ 1.0	9.6 $\pm$ 1.6	12.3 $\pm$ 1.8
	+*/+*	9.0 $\pm$ 2.3	n. d.	15.7 $\pm$ 2.3	16.9 $\pm$ 2.3	12.5 $\pm$ 0.5
LDH IU/10 ⁹ RBC	+/+	4.3 $\pm$ 0.7	5.4 $\pm$ 0.3	3.9 $\pm$ 0.3	4.7 $\pm$ 1.8	3.8 $\pm$ 0.9
	+*/+*	0.6 $\pm$ 0.5	1.7 $\pm$ 0.1	1.4 $\pm$ 0.5	5.9 $\pm$ 0.1	1.1 $\pm$ 0.4

^aData are expressed as mean $\pm$ SD of 3 mice examined at each time point.

^bValues were obtained from non-splenectomized mice examined as controls. All 12 control values (3 at each time point) were combined in order to derive the mean $\pm$ SD.

^cn. d. = not determined

Table 13. Differential counts (in percent) of nucleated cells in peripheral blood of wild type (+/+) and homozygous mutant (+*/+*) mice 16 weeks after splenectomy^a

Parameters	+/+	+*/+*
Total granuloid cells	12.7 ± 1.4	21.0 ± 4.2
Lymphocytes	86.9 ± 1.6	66.7 ± 6.6
Monocytes	---	1.1 ± 0.4
Nucleated red cells	0.4 ± 0.4	10.0 ± 3.6
Unclassified cells	---	1.2 ± 0.3

^aData are expressed as mean ± SD of 3 mice examined; at least 250 cells per mouse were counted.

not shown).

The number of white blood cells was markedly increased in both genotypes (Table 12). These values were about 2 times higher in the wild type and 1.6 times in the mutant mice as compared to values obtained from untreated control mice. Differential counts (Table 13) of the WBC 16 weeks after splenectomy showed that the values in splenectomized wild type mice were almost the same as in untreated mice (Table 4). In splenectomized mutants the fraction of granuloid cells remained almost unchanged, but the appearance of 10% nucleated red cells was striking compared to only 1% in untreated mutants (Table 4). The lymphocytes were diminished by 10%.

The LDH activities in the peripheral blood of splenectomized wild type mice remained within the normal range, whereas in the mutants the values increased by more than 100% (Table 12). From the values of weeks 5, 9 and 16 it can be concluded that there was a tendency towards elevation of LDH activity in the splenectomized homozygous mutants.

In contrast to the untreated controls and splenectomized wild type mice some further remarkable changes in the splenectomized mutants were observed. The lymph nodes were enlarged up to about 3 mm and red in color. Hepatomegaly was not observed but histologically the liver showed several erythropoietic foci. Erythropoiesis has also been observed in the proximal part of the tail bone marrow.

The splenectomized wild type as well as homozygous mutant mice appeared to have a normal life span. Two mice of each genotype survived for 652 days and were then killed for histological investigations.

6.3.2 Cellularity in bone marrow

The numbers of viable nucleated cells per femur in the splenectomized mice of both genotypes are shown in Fig. 4. In the wild type mice the cellularity was almost in the normal range, except for very high values at week 16. More than 50% of the bone marrow cells were found to be dead by the eosin dye-exclusion technique. However, histological investigations of the bone marrow in the vertebral column and the femur of the splenectomized and non-splenectomized mutants showed no difference in cellularity.

6.3.3 Frequencies of CFU-S and GM-CFC

Fig. 5 shows the frequencies of CFU-S and GM-CFC measured at different time points following splenectomy in the femurs of wild type and mutant mice (calculated as percentage of non-splenectomized control mice). In wild type mice, the values for CFU-S were in the normal range throughout, while those in the splenectomized mutants showed a gradual in

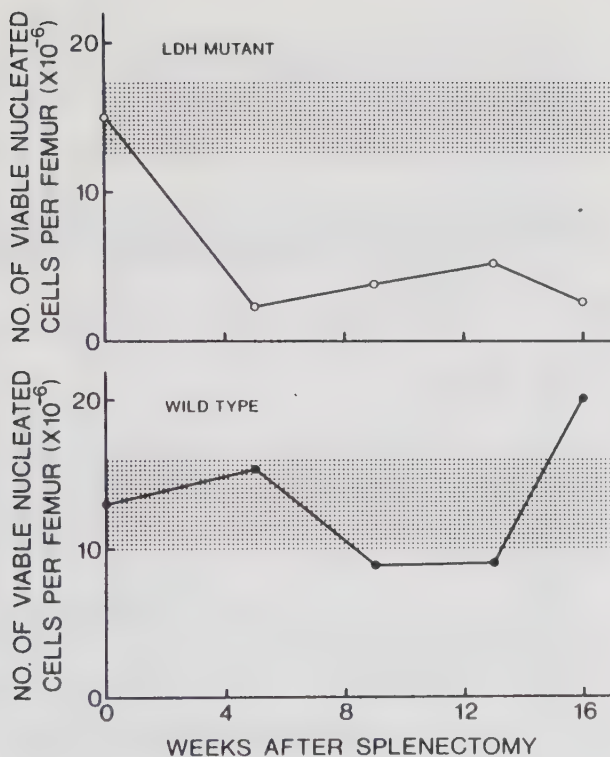


Fig. 4. Number of viable nucleated cells in femurs of splenectomized wild type (closed symbols) and homozygous mutant (open symbols) mice following splenectomy. Each point represents a pool of BMC of 3 mice. Shaded areas show the normal ranges.

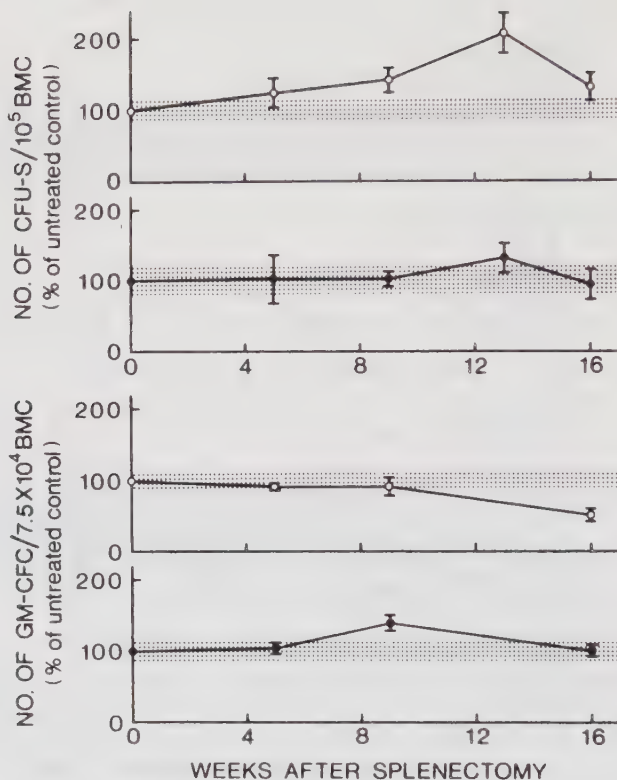


Fig. 5. Frequencies of bone marrow-derived CFU-S and GM-CFC in wild type (closed symbols) and homozygous mutant (open symbols) mice following splenectomy, in percentage of values from non-splenectomized mice. Each point with vertical bars represents the mean $\pm$ SD of values obtained from a pool of BMC of 3 mice. 10^5 BMC were injected into 6 lethally irradiated recipient mice and spleen colonies were counted on day 12. GM-CFC were assayed in 5 replicate cultures by seeding 7.5×10^4 BMC/plate, stimulated with 0.2 ml MLCM/plate.

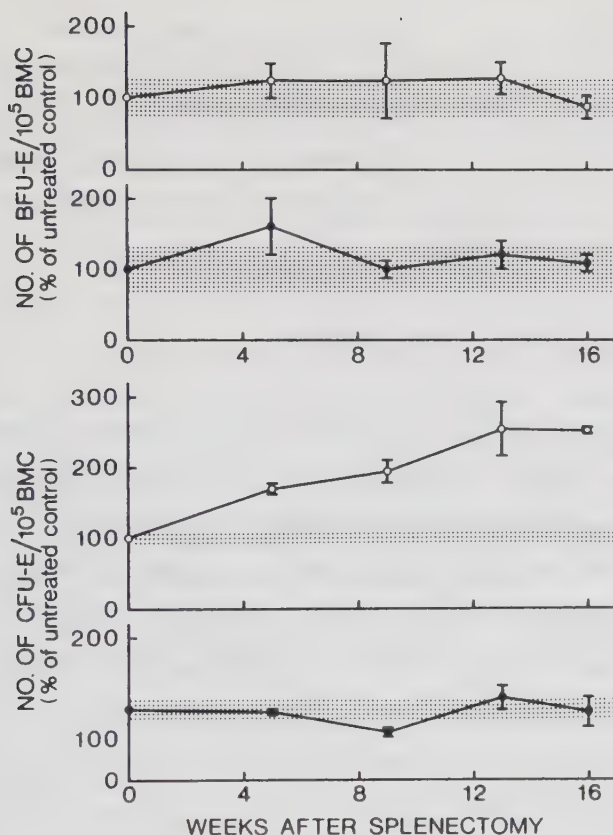


Fig. 6. Frequencies of bone marrow-derived BFU-E and CFU-E in wild type (closed symbols) and homozygous mutant (open symbols) mice following splenectomy, in percentage of values from non-splenectomized mice. Each point with vertical bars represents the mean $\pm$ SD of values obtained from a pool of BMC of 3 mice. BFU-E and CFU-E were assayed in parallel performing 5 replicate cultures for each. 10^5 BMC/plate were stimulated with SCM (0.2 ml/plate) and EPO (1.5 units/plate) for BFU-E and only EPO (1 unit/plate) for CFU-E.

crease with a peak at week 13, and then a decline towards normal values at week 16.

The frequency of GM-CFC in the femur of splenectomized wild type mice was in the normal range except for a rise at week 9 (about 140% of control value). The GM-CFC frequency in the splenectomized mutants showed a fall (about 50% of control) at week 16, otherwise the values were in the normal range.

6.3.4 Frequencies of BFU-E and CFU-E

The frequencies of BFU-E and CFU-E in the femurs of splenectomized mice are shown in Fig. 6. The values for BFU-E remained in the range of controls in both genotypes. The frequencies of CFU-E in the wild type bone marrow were also in the normal range. The gradual increase of femoral CFU-E frequencies in the splenectomized mutants was very remarkable, reaching 250% of the control values at week 13 and persisting at this level until week 16.

6.3.5 Discussion

The series of observations made in the homozygous mutant following splenectomy reveals first of all the important fact that splenic contribution to total hemopoiesis is not indispensable for their survival.

It is generally known that in states of hypersplenism, i.e., when the spleen has an increased activity of erythrocyte sequestration, splenectomy may raise the hematocrit (Wintrobe et al., 1981). Splenomegaly is also present in other inherited hemolytic anemias of the mouse (Bernstein, 1980). In these cases, splenectomy could not raise the

hematocrit, rather, the hematocrit declined gradually, causing death of the mice. The author concluded that splenomegaly in these mutants was the result of an increased erythrocyte sequestering activity as well as a of a highly expanded erythropoiesis. In the splenectomized LDH mutant, conditions found in peripheral blood lead to the same conclusion. The constantly low RBC count emphasizes the fact that the production of RBC by the spleen is lacking. One possible reason for the progressively decreasing fraction (from about 95% to about 80%) of reticulocyte is: the splenic destruction of erythrocytes is absent, and consequently some erythrocytes may survive for a longer period of time, thus lowering the fraction of reticulocytes in the blood. The simultaneous increase of poikilocytes and anisocytes supports this argument. Moreover, the increase of LDH activity in RBC (Table 12) is very striking. The increased mean cellular volume may be a reason for this.

The splenectomized wild type mice showed an anemia, a post-splenectomy effect which has also been observed by Lozzio (1972), who compared various hematological parameters of the congenetically asplenic heterozygous (Dh/+), normal (+/+) and splenectomized normal mice. Anemia has also been found in some strains of mice and rats following splenectomy (Bozzini et al., 1970; Crosby, 1963; Palmer et al., 1951). The increased number of reticulocytes correlates with the mild anemia observed in the present splenectomized wild type mice (Table 12).

In the normal mouse, liver erythropoiesis is found only in embryonal stage (Metcalf and Moore, 1971) and in the tail bone marrow no hemopoiesis takes place. However, extramedullary hemopoiesis in the liver has been documented in non-splenectomized mutant mice with spherocytic hemolytic anemia (Unger et al., 1983) and after infection with Friend viruses (Seidel and Kreja, 1983). The presence of functionally hemo-

poietic tissue in the liver of the splenectomized LDH mutants suggests that reactivation of extramedullary hemopoietic sites takes place to maintain a steady state in the mouse. The lymph nodes of the splenectomized mutants were enlarged. From the histological findings, extramedullary hemopoiesis did not prove to be a reason for the enlargement of the lymph nodes.

Following preparation of bone marrow cells of the splenectomized mutants, more than 50% of the cells were found to be dead according to the eosin dye exclusion test. Since histological investigations revealed no difference between splenectomized and non-splenectomized mutants, it can be concluded that due to some unknown reason these cells were extremely fragile. Further investigations are needed to explain this unusual phenomenon. Nevertheless, it is assumed that the remaining viable nucleated cells were representative of the original population in the bone marrow. The increase in the frequency of CFU-S at week 13, and especially the elevated frequency of CFU-E may be attributed to two reasons: firstly, in non-splenectomized mutants stem cells and late erythroid precursors could migrate from the bone marrow into the spleen (Hodgson, 1985; Rencricca et al., 1970); secondly, erythropoiesis in the bone marrow is stimulated in order to compensate for the lack of splenic hemopoiesis.

6.4 Proliferation kinetic studies

The proliferative status of the hemopoietic stem cells and progenitors were determined by applying the ^3H -TdR suicide technique (see 5.14). In order to investigate the proliferation kinetics of the morphologically recognizable erythroid compartments in the spleen, labeling indices as well as the duration of DNA synthesis were estimated (see 5.13) using wild type and homozygous mutant mice.

6.4.1 Cell cycle status of hemopoietic stem cells and progenitors

CFU-S in the bone marrow and spleen of both genotypes showed almost no measurable kill, indicating that the CFU-S are not triggered into the cell cycle, even in a permanent state of erythropoietic stress in the mutants (Table 14). No genotypic difference was observed in the suicide rates of the progenitor compartments investigated. A high percentage of kill (up to about 80%) could be measured in the CFU-E compartment of the mutants as well as of the wild type mice.

6.4.2 Proliferation kinetics of the morphologically recognizable erythroblast compartments

The DNA synthesis time investigated in the splenic erythroblast compartments of the wild type and homozygous mutant mice is shown in Fig. 7. The values increased with progressing maturation, i.e., from proerythroblasts towards polychromatic erythroblasts. The duration of the S-phase was significantly shorter in the basophilic and polychromatic erythroblast compartments of the mutants when compared to that of the

Table 14. Cell cycle status of hemopoietic stem and progenitor cells in wild type (+/+) and homozygous mutant (+*/+*) mice assayed by in vitro tritiated thymidine suicide^a

Cells assayed	% kill			
	in bone marrow		in spleen	
	+/+	+*/+*	+/+	+*/+*
CFU-S ^b				
Experiment 1	7	7	-4	-2
Experiment 2	-5	0	-12	-17
GM-CFC ^c				
Experiment 3	23	27	37	36
Experiment 4	31	22	27	42
Experiment 5	18	25	15	27
BFU-E ^d				
Experiment 6	25	20	25	40
Experiment 7	56	50	50	20
Experiment 8	18	20	56	50
CFU-E ^d				
Experiment 6	60	77	82	60
Experiment 7	68	75	82	73
Experiment 8	67	73	79	70

^aCell suspensions pooled from 3 or 4 donors of both sexes were used.

^bEach cell suspension was injected into 6 lethally irradiated recipients (see 5.9).

^cCells were assayed in 5 replicate agar cultures (see 5.10.1).

^dCells were assayed in 5 replicate methylcellulose cultures (see 5.10.2).

wild type mice ($P < 0.001$, determined by Student's t-test).

The labeling indices determined from the same animals tended to be slightly higher in the mutants, although only the value of polychromatic erythroblast was significantly different ($P < 0.01$, determined by Student's t-test) (Fig. 7).

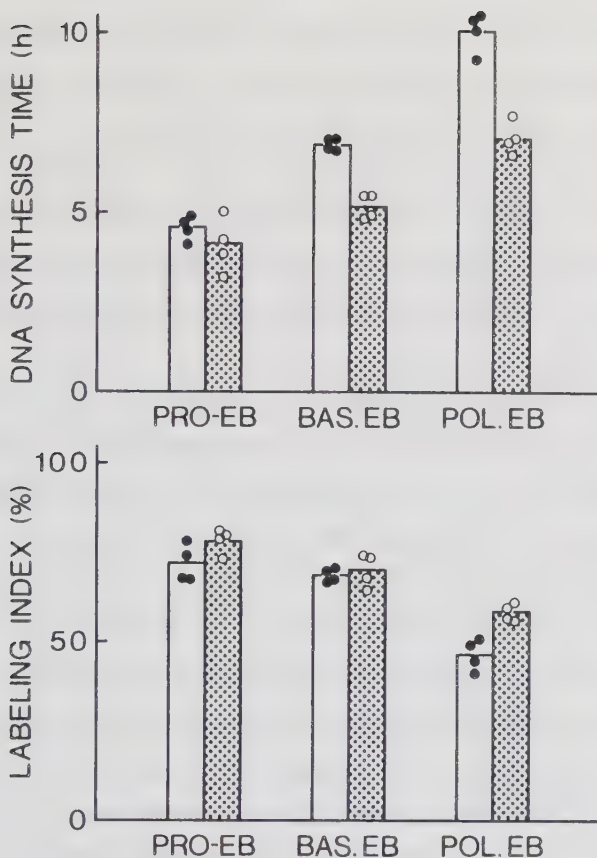


Fig. 7. DNA synthesis time and labeling index in morphologically recognizable splenic erythroblast compartments of wild type (closed symbols, free columns) and homozygous mutant (open symbols, dotted columns) mice. DNA synthesis time was determined by quantitative ^{14}C -autoradiography and labeling index by ^3H -TdR labeling. At least 50 labeled proerythroblasts (Pro-EB), 100 basophilic (Bas. EB) and 100 polychromatic erythroblasts (Pol. EB) were evaluated per mouse. Individual values and mean values obtained from 4 mice in each genotype are presented.

6.4.3 Discussion

The cell cycle status of the hemopoietic stem cells and progenitors was not affected by the stress situation due to hemolytic disease. In particular, the fraction of cells in S-phase was not significantly increased either in the early (BFU-E) or in the late (CFU-E) erythroid progenitor compartment. Comparable results were obtained from the investigations of BFU-E and CFU-E compartments in mice with phenylhydrazine-induced hemolytic anemia or after bleeding (Hara and Ogawa, 1977; Iscove, 1977)

The duration of DNA synthesis in the basophilic and polychromatic erythroblast compartments in mutants was significantly reduced (Fig. 7). In addition, a significantly elevated percentage of labeled polychromatic erythroblasts was found. Dörmer (1981) described a similar reduction of DNA synthesis time in marrow erythroblasts of phenylhydrazine-treated rats. Shortening of the cell cycle time was found by Hanna et al. (1969) in the erythroid precursors of rats subjected to protracted anemia by phenylhydrazine injection. Since the duration of DNA synthesis in morphologically recognizable erythroid precursors is shortened in the present case, it can be assumed that a faster maturation from CFU-E to erythrocytes occurs (also observed by Tarbutt (1969) in protracted anemia). Moreover, it could be calculated from the total body content values for CFU-E and BFU-E, that approximately 4 additional cell divisions (see 5.11) must occur in the differentiation pathway from BFU-E to CFU-E.

6.5 Production of lymphokines by mitogen-stimulated spleen cells

Murine spleen cells in culture stimulated with PHA, PWM, Con A or bacterial lipopolysaccharide produce large amount of CSF (Parker and Metcalf, 1974). Of the four murine CSFs stimulating granulocyte-macrophage colonies (Metcalf, 1986a) two --- GM-CSF and multi-CSF or (IL 3) --- are secreted by lectin-stimulated spleen cells (Metcalf, 1984).

In pilot studies we found that in the homozygous mutants SCM produced following stimulation with PWM (PWM-SCM) showed only a minimum detectable amount of CSF (tested by the semisolid agar assay, 5.10.1) under the standard conditions used in our laboratory (2×10^6 SPC/ml; harvesting day 7). It was assumed that the mutants might have a qualitative or quantitative defect with respect to those cells involved in the secretion of lymphokines. In order to investigate the phenomenon in detail, three mitogens (PHA, PWM, Con A) were selected. Optimum concentrations (PHA 1%, PWM 5%, Con A 5 μ g/ml) worked out in preliminary experiments were added to the spleen cell cultures from either the wild type or the mutant mice. In order to study the kinetics of lymphokine production all three types of SCM (named according to the mitogen used PHA-SCM, PWM-SCM, Con A-SCM) were harvested on days 3, 5 and 7, and three different cell concentrations were used.

6.5.1 CSF-assay

The levels of CSF detected in different SCM of wild type and homozygous mutant mice following stimulation with different mitogens are summarized in Fig. 8.

CSF present in the PHA-SCM from wild type cells reached a plateau

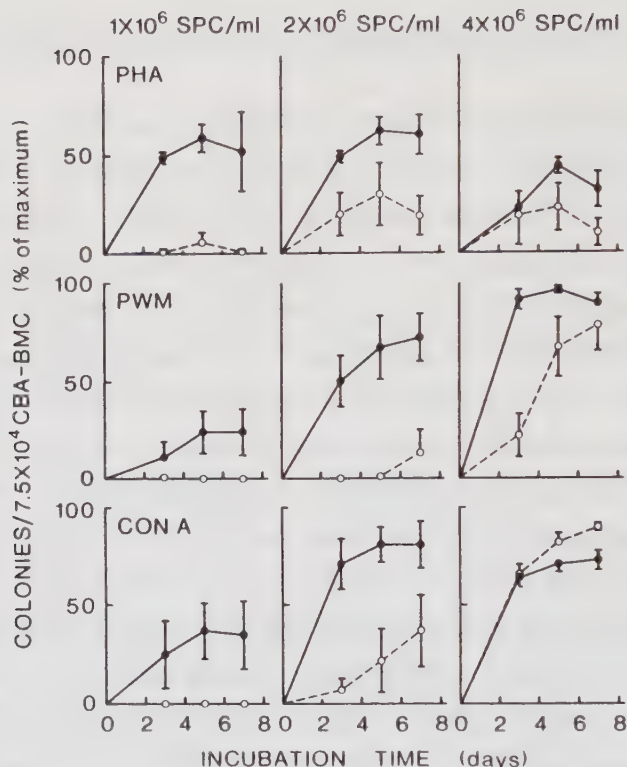


Fig. 8. Kinetics of production of CSF by spleen cells (SPC) stimulated with different mitogens (PHA, PWM, Con A) in wild type (closed symbols, full lines) and homozygous mutant (open symbols, dashed lines) mice. The biological activity of the supernatants (0.2 ml/plate) was tested in triplicate cultures against CBA-BMC 7.5×10^4 /plate. Percentages of maximum were calculated for each experiment. The maximum number of GM-CFC colonies in the three experiments were 115, 70, 102, respectively. Each point with vertical bars represents the mean $\pm$ SEM of three experiments. No CSF activity could be measured in SCM produced in the absence of mitogens.

level after 5 days with 1×10^6 and 2×10^6 SPC/ml. The level was comparatively low with 4×10^6 SPC/ml. Conversely, in the mutants, with 1×10^6 SPC/ml, the level of CSF present was very low. With 2×10^6 SPC/ml there was a relatively higher level of CSF present, but in general, the level was much lower than with wild type spleen cells.

There was a positive correlation between the number of cells initially seeded and the CSF concentration in PWM-SCM of the wild type. With 4×10^6 SPC/ml the production had reached a plateau already on day 3. In the mutants, there was practically no detectable CSF production with 1×10^6 SPC/ml, and with 2×10^6 SPC/ml only a little production was detectable on day 7. With 4×10^6 SPC/ml the slope of CSF level was low until day 3, then increased significantly until day 5 and then reached a level comparable to the wild type value at day 7.

According to the kinetics of CSF production in Con A-SCM of wild type mice the plateau level of CSF was observed on day 5 (Fig. 5). A detectable amount of CSF was produced with 1×10^6 SPC/ml. The production was significantly enhanced with 2×10^6 SPC/ml, but was not further increased with 4×10^6 SPC/ml. The spleen cells of the mutants, on the contrary, produced no detectable CSF at 1×10^6 SPC/ml. The curve of CSF level with 2×10^6 SPC/ml rose constantly but the quantity was very low even at day 7. Quite remarkable was the production of CSF with 4×10^6 mutant SPC/ml, which slightly overshot the values in wild type.

No CSF activity could be measured in spleen cell conditioned media produced in the absence of mitogens in spleen cell cultures of both genotypes.

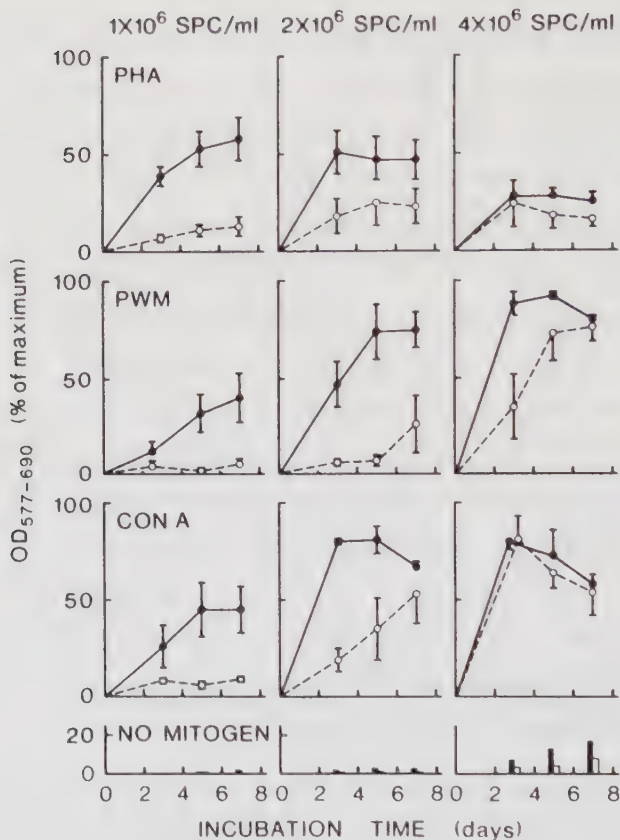


Fig. 9. Kinetics of production of IL-3 by spleen cells (SPC) stimulated with different mitogens (PHA, PWM, Con A) in wild type (closed symbols, full lines) and homozygous mutant (open symbols, dashed lines) mice. The biological activity of the supernatants was tested in quadruplicate cultures in 96-well plate (2×10^4 cells/100 μ l/plate; supernatant 10 μ l/plate) against a IL-3 dependent mast cell line. Percentages of maximum were calculated for each experiment. The maximum OD₅₇₇₋₆₉₀ values for the three experiments were 0.887, 0.897 and 1.047, respectively. Each point with vertical bars represents the mean $\pm$ SEM of three experiments.

6.5.2 Interleukin 3 assay

The kinetics of the production of interleukin 3 by the spleen cells of wild type and mutant mice after stimulation with different mitogens are shown in Fig. 9.

IL-3, which is synonymous with Multi-CSF, was assayed by measuring the growth of a permanent IL-3 dependent mast cell line established in our laboratory. This was performed by a using colorimetric method (see 5.8).

In the PHA-SCM of wild type mice IL-3 reached a plateau level already on day 3 in cultures containing 2×10^6 SPC/ml and 4×10^6 SPC/ml. IL-3 activity in cultures containing 1×10^6 SPC/ml increased with each day. In mutants the activities were altogether lower than in the wild type, and cultures containing 2×10^6 SPC/ml showed the highest values.

The profiles of IL-3 activities measured in PWM-SCM of both genotypes were very similar to those shown for CSF (Fig. 8). The wild type curve with 2×10^6 SPC/ml was similar to the mutant curve with 4×10^6 SPC/ml. It was evident from both bioassays that roughly double the number of cells was required in the mutants to get the same response, and the production was even delayed until day 7.

IL-3 activity detected in Con A-SCM of wild type was highest when 2×10^6 or 4×10^6 SPC/ml were seeded, but there was a decline after 5 and 3 days, respectively. The spleen cells of the mutants produced only minimum activity at 1×10^6 SPC/ml, while 2×10^6 SPC/ml produced significant amounts of IL-3 gradually increasing day by day. The activities produced by 4×10^6 SPC/ml showed almost the same course as in the wild type.

SCM without mitogen showed only a very low amount of IL-3 activity in cultures containing 1 or 2×10^6 SPC/ml. With 4×10^6 SPC/ml the background activity was relatively higher, especially in the wild type.

6.5.3 Discussion

The spleen is composed of different kinds of cells such as T and B lymphocytes, macrophages and monocytes. These may have a direct (producer) or indirect (accessory) role in the production of lymphokines following stimulation with different mitogens. In the mouse, PHA and Con A stimulate T cells selectively (Janossy and Greaves, 1972; Stobo et al., 1972; Unanue and Benacerraf, 1984) and the cells responding to PHA and Con A presumably belong to different subsets of T lymphocytes. Parker and Metcalf (1974) suggested that macrophages might not be involved in the response to PHA, while response to Con A is macrophage-dependent. In the latter case, at least the presence of macrophage-conditioned supernatant is required which partially replaces them (Unanue and Benacerraf, 1984). Response to PWM appeared to involve both T and B lymphocytes (Shortman et al., 1973; Parker and Metcalf, 1974). With the help of established T cell lines it has been demonstrated that different T cell clones have the capability to produce different lymphokines (Watson, 1983; Koyasu et al., 1985; Kelso et al., 1986).

In a comparison of the spleen cell conditioned media of wild type and mutant mice, it becomes evident that 4×10^6 SPC/ml of mutants are necessary to get approximately the same amount of CSF production as produced by 2×10^6 SPC/ml of the wild type mice.

It can be suggested that there is a quantitative difference between different spleen cell populations (e.g., erythroid, lymphoid) of the

wild type and the mutant mice. In mutants, the quantitative deficiency of cells involved in lymphokine production is presumed to be the main reason for the low production of different lymphokines. It is evident from the differential count (Table 6) of the morphologically recognizable precursors in the spleen that in mutants only 23% are lymphoid cells in contrast to 77% in the wild type mice. Moreover, more than 65% of the spleen cell population belongs to the erythroid series, whereas the value is only 9% in the wild type. It is unlikely that the T cells of the mutants are hyporesponsive to those mitogenic stimuli, because optimum concentrations of different mitogens were found to be similar in both genotypes. In general, it is likely that the same number of producer and accessory cells in mutant and wild type mice produce comparable amounts of growth factors.

6.6 Test of sera for the presence of CSF and EPO

Crude sera of wild type and homozygous mutant mice were assayed for the presence of CSF and EPO. The CSF content was determined using the semisolid agar assay (see 5.10.1). Serial dilutions of sera or MLCM (control) were used as stimulus. No colonies could be detected in the plates containing mouse sera.

The content of EPO in sera was estimated using the semisolid methylcellulose assay (see 5.10.2) for CFU-E colony formation (Fig. 10). Serial dilutions of serum or standard step III EPO (control) were used as stimulus. Colony formation in the presence of serum alone was compared with the colony count in the presence of step III EPO. The control value was 292 ± 5 CFU-E/ 10^5 BMC of the homozygous mutant mouse. Both wild type and mutant sera showed almost the same dose response curve, the highest colony yield being 15% of control at a concentration of 2.5% serum/ plate. This amount corresponds to approximately 0.1 unit EPO/ml of serum in both genotypes.

6.6.1 Discussion

CSF has been detected in fetal mouse serum. In the adult mouse, on the contrary, it is very low (Johnson and Metcalf, 1978). The serum CSF level increases significantly following infection, which has been shown by several authors after injecting bacterial cell wall complexes intravenously (Metcalf, 1971; Quesenberry et al., 1972; Metcalf, 1980).

The hormone erythropoietin is produced in the kidney in response to low oxygen tension (Wintrobe et al., 1981). It is found in the plasma and urine of animals experimentally made hypoxic as well as of anemic

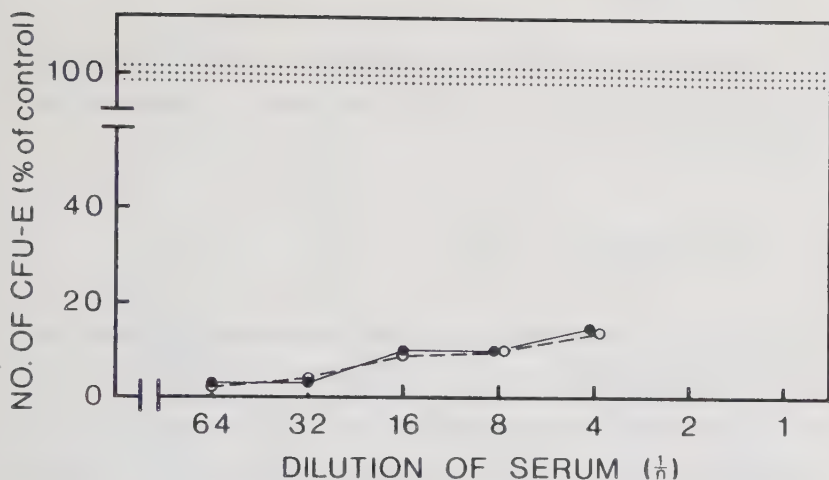


Fig. 10. Serial dilutions of crude serum of wild type (closed symbols, full lines) and homozygous mutant (open symbols, dashed lines) mice tested against 10^5 BMC of the homozygous mutant per plate to estimate the amount of EPO present by CFU-E colony formation. The control was performed with Connaught EPO step III and the 100% colony count was $292 \pm 5/10^5$ BMC. Each point represents the mean of triplicate cultures. Standard deviations were less than 1% of control.

patients (Epsada, 1977). In the present experiment no elevation of serum EPO level was detected in the mutants with hemolytic anemia. This finding corresponds to those made by Erslev and Silver (1975) in rat with experimentally induced anemia.

In order to explain the efforts in the LDH mutants to compensate for the anemia, it has to be assumed that either there is a more efficient oxygen carrier system (for which there is no evidence) or that the turnover of EPO is increased so the level of EPO in serum remains normal.

6.7 Radiosensitivity

The LDH mutants turned out to be extremely radiosensitive. Several experiments were done to find out the cause of this increased radiosensitivity.

6.7.1 Lethal dose_{50/30} (LD_{50/30})

Groups of wild type and homozygous mutant mice received different doses of total body irradiation (TBI). The doses were 6.75, 7.00, 7.25, 7.50 or 7.75 Gy for the wild type, and 4.00, 4.25, 4.50, 4.75 or 5.00 Gy for the mutant mice. The values of LD_{50/30} for wild type and homozygous mutant mice were calculated from the probit of percentage mortality of mice of both genotypes plotted against the log of irradiation dose (Fig. 11). The LD_{50/30} for the wild type mice was 7.3 Gy and for mutants 4.4 Gy.

6.7.2 D₃₇ values for hemopoietic stem and progenitor cells

The D₃₇ values for the hemopoietic stem and progenitor cells in both genotypes were determined in order to explain whether the increased radiosensitivity of the mutant mice was related to an increased radiosensitivity in these compartments.

6.7.2.1 D₃₇ values for CFU-S

The surviving fraction of CFU-S in a semilogarithmic plot against the irradiation dose is shown in Fig. 12. In each genotype, five aliquots

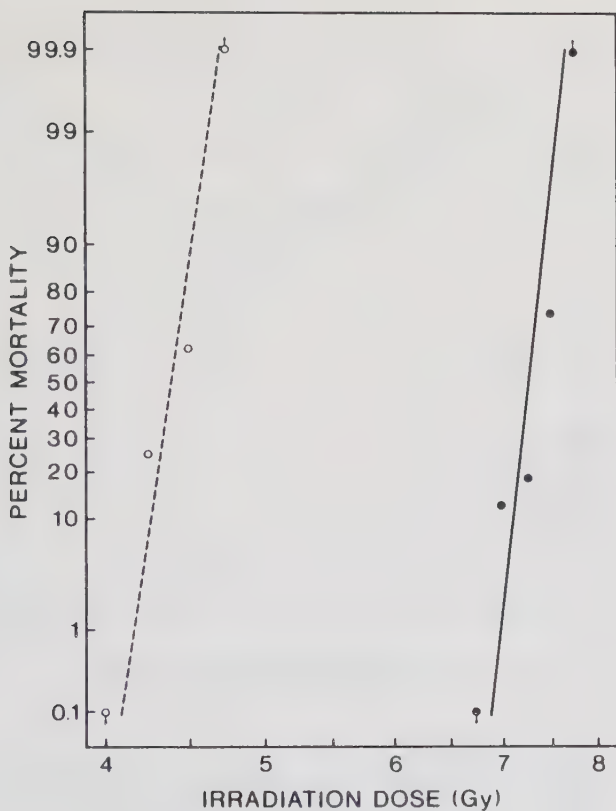


Fig. 11. Probit of percentage mortality in wild type (closed symbols, full line) and homozygous mutant (open symbols, dashed line) mice plotted against the log of the radiation dose. In each genotype, 5 groups of mice, each consisting of 16 mice, were irradiated with 5 different doses (see 6.7.1) from a caesium-137 source at a rate of 1.15 Gy/min. LD_{50/30} values for wild type and mutant mice were 7.3 Gy and 4.4 Gy, respectively.

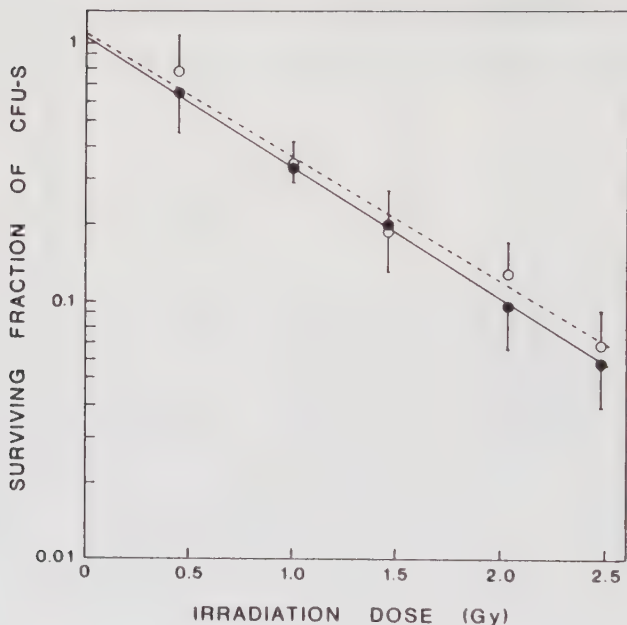


Fig. 12. Survival curves of CFU-S from in vitro irradiated bone marrow cells of wild type (closed symbols, full line) and homozygous mutant (open symbols, dashed line) mice. Five aliquots of bone marrow cell suspension pooled from 4 donors were irradiated with 5 different doses (see 5.15.2) from a caesium-137 source at a rate of 1.13 Gy/min and each one was injected into 6 lethally irradiated normal recipients. Non-irradiated cells were treated in the same way as controls. CFU-S of wild type and mutant mice showed a D_{37} value of 0.90 ± 0.04 Gy (extrapolation no. 1.06) and 0.98 ± 0.12 Gy (extrapolation no. 1.10), respectively. Colonies were counted on day 10. Data are expressed as mean $\pm$ SD of one experiment.

of bone marrow cell suspension pooled from four donors were irradiated in vitro with five different doses (see 5.15.2). The CFU-S assay was performed with each aliquot (see 5.9) by injection into six recipients. The values of D_{37} (mean $\pm$ SD) were 0.90 ± 0.04 Gy (extrapolation number 1.06), and 0.98 ± 0.12 Gy (extrapolation number 1.10) for wild type and mutant mice, respectively.

6.7.2.2 D_{37} values for GM-CFC

Fig. 13 shows the survival curve of GM-CFC in both genotypes. In each genotype BMC pooled from 2-4 mice were irradiated (see 5.15.2) in vitro. Three separate experiments were performed. Irradiated cells were subsequently assayed for GM-CFC in triplicate cultures stimulated by MLMC (see 5.10.1). The D_{37} value for wild type was 1.47 ± 0.29 Gy (extrapolation number 0.97) and that for the mutant mice was 1.67 ± 0.30 Gy (extrapolation number 0.95).

6.7.2.3 D_{37} values for BFU-E and CFU-E

Figs. 14 and 15 show the survival curves of BFU-E and CFU-E, respectively, in wild type and mutant mice. Aliquots of bone marrow cell suspension (pooled from 7-8 mice) were irradiated (see 5.15.2) and assayed for BFU-E and CFU-E, each in 5 replicate cultures stimulated with BPA and/or EPO as needed (see 5.10.2). The wild type mice had a D_{37} value of 1.08 ± 0.36 Gy (extrapolation number 0.92) and 1.03 ± 0.38 Gy (extrapolation number 0.90) for BFU-E and CFU-E, respectively. The mutants showed a D_{37} value of 1.04 ± 0.38 Gy (extrapolation number 0.90) and 1.10 ± 0.33 Gy (extrapolation number 0.93) for BFU-E and CFU-

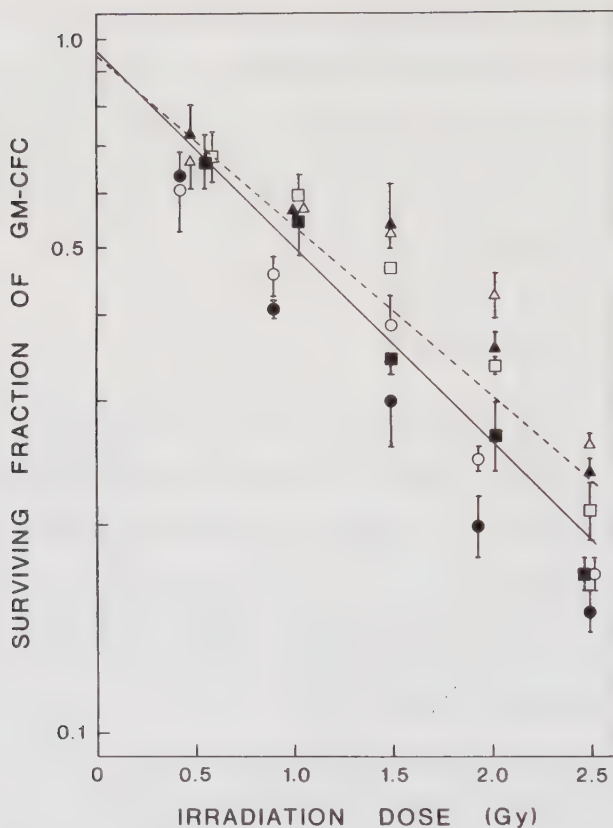


Fig. 13. Survival curves of GM-CFC from in vitro irradiated bone marrow cells of wild type (closed symbols, full line) and homozygous mutant (open symbols, dashed line) mice. Five aliquots of bone marrow cell suspension pooled from 2-4 mice were irradiated with 5 different doses (see 5.15.2) from a caesium-137 source at a rate of 1.13 Gy/min. Cells were subsequently assayed for GM-CFC in triplicate cultures stimulated with MLCM. The D_{37} values for wild type and mutant mice were 1.47 ± 0.29 Gy (extrapolation no. 0.97) and 1.67 ± 0.30 Gy (extrapolation no. 0.95), respectively. Data are presented as mean $\pm$ SD of 3 separate experiments.

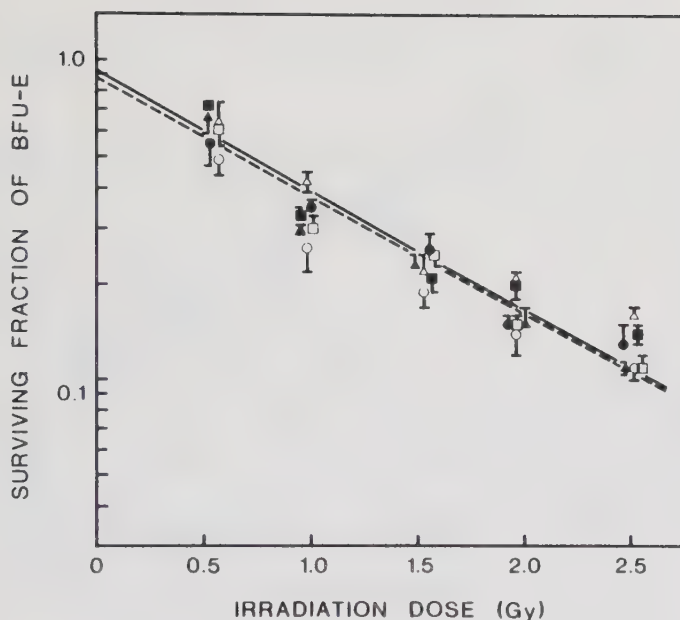


Fig. 14. Survival curves of BFU-E from in vitro irradiated bone marrow cells of wild type (closed symbols, full line) and homozygous mutant (open symbols, dashed line) mice. Five aliquots of bone marrow cell suspension pooled from 7-8 mice were irradiated with 5 different doses (see 5.15.2) from a caesium-137 source at a rate of 1.09 Gy/min. Cells were subsequently assayed for BFU-E in triplicate cultures stimulated with WEHI-3B-CM and EPO. The D_{37} values for wild type and mutant mice were 1.08 ± 0.36 Gy (extrapolation no. 0.92) and 1.04 ± 0.38 Gy (extrapolation no. 0.90), respectively. Data are presented as mean $\pm$ SD of three separate experiments.

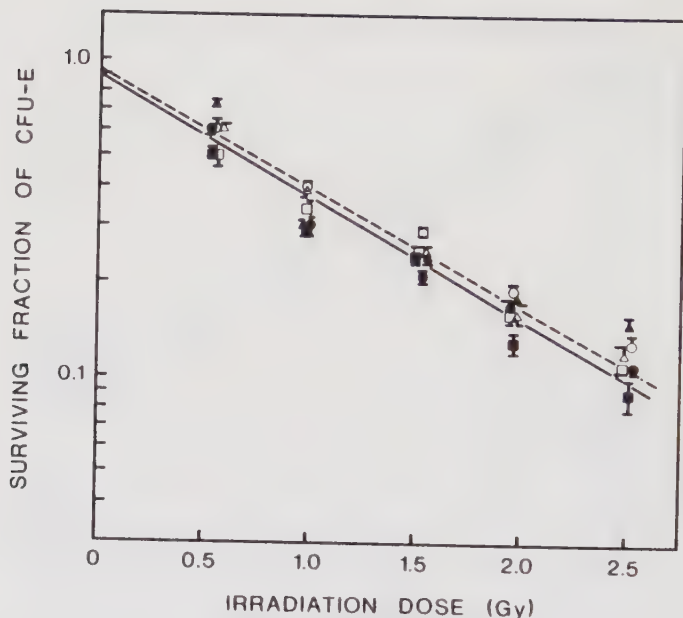


Fig. 15. Survival curves of CFU-E from in vitro irradiated bone marrow of wild type (closed symbols, full line) and homozygous mutant (open symbols, dashed line) mice. Five aliquots of bone marrow cell suspension pooled from 7-8 mice were irradiated with 5 different doses (see 5.15.2) from a caesium-137 source at a rate of 1.09 Gy/min. Cells were subsequently assayed for CFU-E in triplicate cultures stimulated with EP0. The D_{37} values for wild type and mutant mice were 1.03 ± 0.38 Gy (extrapolation no. 0.09) and 1.10 ± 0.33 Gy (extrapolation no. 0.93), respectively. Data are presented as mean $\pm$ SD of 3 separate experiments.

E, respectively.

6.7.3 Observations after irradiation with 3.0 Gy

Wild type and homozygous mutant mice were examined for different parameters following TBI with 3.0 Gy to detect whether in mutants a slower process of hemopoietic regeneration after irradiation is responsible for the extreme radiosensitivity.

Mice of both genotypes were sublethally irradiated with 3.0 Gy. Three irradiated and three untreated control mice were examined individually at each time for peripheral blood values. In each of those two groups BMC and SPC were pooled from 3 mice for quantification of hemopoietic stem and progenitor cells. The days of examination were 1, 4, 7, 14 and 29.

6.7.3.1 Recovery pattern of peripheral blood values and spleen weights following 3.0 Gy TBI

The time course for the recovery of RBC and reticulocytes is shown in Fig. 16. In mutants, the RBC count fell to a remarkable nadir on day 4, and then rose. In wild type mice, on day 4 a slight fall was observed. On day 7 the values reached about 100% and remained stable in wild type mice for the rest of the period. The mutants showed a short depression in the curve at day 14 and by day 29 the RBC count was normal. In wild type mice, at day 4 the reticulocytes were largely diminished, at day 14 the number was slightly higher than normal and they reached the normal range by day 29.

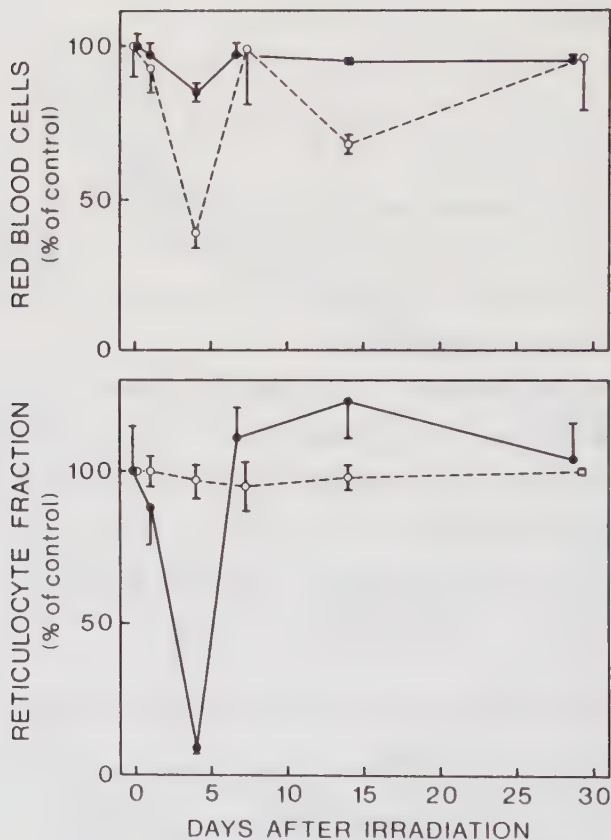


Fig. 16. Patterns of recovery of red blood cells and reticulocytes following 3.0 Gy TBI in the wild type (closed symbols, full lines) and homozygous mutant (open symbols, dashed lines) mice. Each point with vertical bar represents the mean $\pm$ SD of values from 3 mice in percentage of the controls.

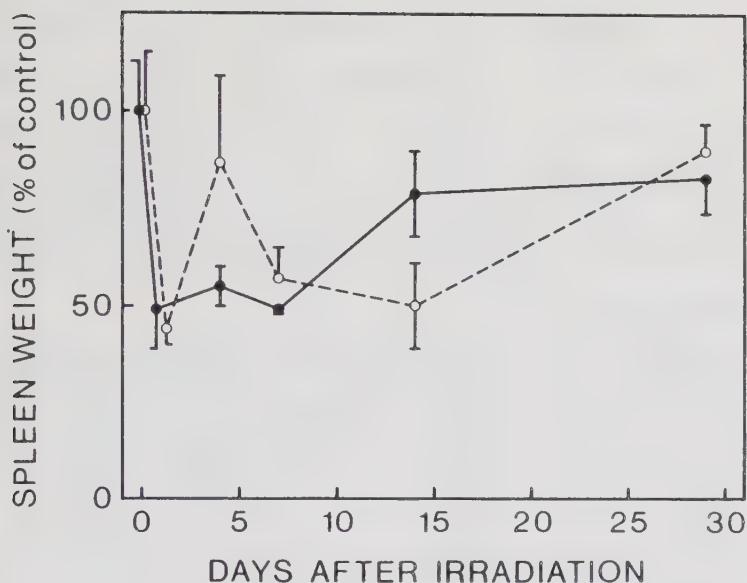


Fig. 17. Patterns of recovery of spleen weight following 3.0 Gy TBI in the wild type (closed symbols, full lines) and homozygous mutant (open symbols, dashed lines) mice. Each point with vertical bar represents the mean $\pm$ SD of values from 3 mice in percentage of control.

The spleen weights in both genotypes decreased drastically in the first day after irradiation (Fig. 17). In wild type mice, it remained at a lower level until day 7 and then tended towards normal. In mutants, there was an abortive rise on day 4 followed by a second fall. By day 29 the values in both genotypes were in the range of untreated controls.

6.7.3.2. Recovery patterns of CFU-S following 3.0 Gy TBI

Fig. 18 summarizes the patterns of recovery of CFU-S in femurs and spleens of both genotypes. 'Post-irradiation dip', i.e., a sudden fall in the frequency as well as the number of femoral CFU-S as a consequence of irradiation was observable in both cases. Both the frequency and the number of the femoral CFU-S remained lower in mutants than in wild type mice until day 14, indicating a delayed repopulation of CFU-S. By day 29 femoral CFU-S of both genotypes recovered to the range of untreated controls.

In spleens of both genotypes the course of recovery was almost the same, except that the wild type showed no detectable CFU-S in the spleen at days 4 and 7, while the mutants showed the same result only at day 7.

6.7.3.3 Recovery patterns of GM-CFC following 3.0 Gy TBI

Fig. 19 shows the recovery patterns of GM-CFC in femurs and spleens of both genotypes. During the first 4 days the slope of repopulation in the mutant femur declined, whereas that in the wild type remained at a higher level. Thereafter the femur GM-CFC population in the mutant rose on day 7 followed by a very sharp decline. In the wild type, almost a steadily increasing regeneration was observed both in femurs and spleens. In the mutants, the recovery was staggered in comparison to the wild type. By day 29 GM-CFC of both genotypes reached the range of untreated controls.

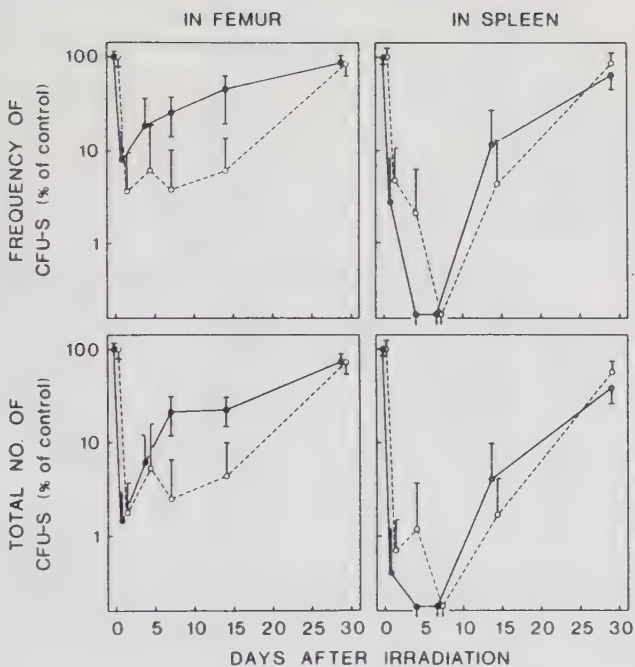


Fig. 18. Patterns of recovery of femoral and splenic CFU-S following 3.0 Gy TBI in the wild type (closed symbols, full lines) and homozygous mutant (open symbols, dashed lines) mice. In each group, including non-irradiated controls, BMC and SPC were pooled from 3 mice and each cell suspension was injected into 6 lethally irradiated recipients (see 5.9). Colonies were counted at day 12. Each point with vertical bars represents the mean $\pm$ SD in percentage of control.

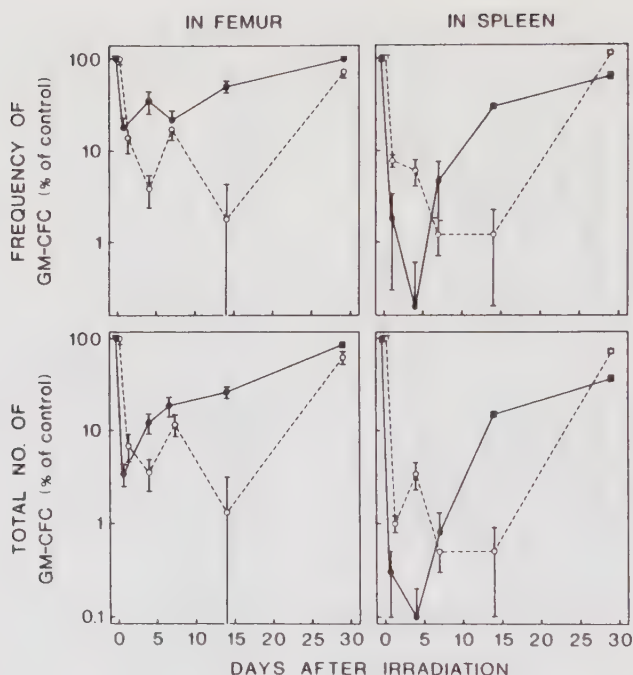


Fig. 19. Patterns of recovery of femoral and splenic GM-CFC following 3.0 Gy TBI in wild type (closed symbols, full lines) and homozygous mutant (open symbols, dashed lines) mice. In each group, including non-irradiated controls, BMC and SPC were pooled from 3 mice and each cell suspension was assayed for GM-CFC in 5 replicate cultures stimulated with MLCM (see 5.10.1). Each point with vertical bars represents the mean $\pm$ SD in percentage of control.

6.7.3.4 Recovery patterns of BFU-E and CFU-E following 3.0 Gy TBI

The recovery patterns of erythroid progenitors are shown in Figs. 20 and 21. In femurs the slopes of regeneration of BFU-E and CFU-E initially displayed similar patterns in both genotypes. After 7 days the regenerations in the mutants slowed, while those of the wild type were unchanged. At day 29 the BFU-E values of both genotypes were in the range of untreated controls. However, in both genotypes the CFU-E values reached more than twice the control levels.

In the spleens, the regeneration patterns of BFU-E were similar in both genotypes until day 7. Then the wild type BFU-E showed a steady process of regeneration. In mutants, on the contrary, the BFU-E population showed a drop on day 14. By day 29 the frequencies were in the range of controls in both genotypes. However, the total number in the spleens remained below the level of controls due to the low cellularity. The splenic CFU-E in the wild type showed a lower 'post-irradiation dip' than in the mutant mice and an oscillating regeneration curve. The frequency in the wild type was about 3 times higher than the control level at days 4 and 14, respectively, and was at a subnormal level (30% of control) on day 7. On day 29, the frequency as well as the total number of splenic CFU-E were higher than control values in both genotypes, except for the total number in spleens of the mutants which was in the normal range.

6.7.3.5 Cellularities in femurs and spleens following 3.0 Gy TBI

The cellularities (Table 15) in femurs was subnormal until day 14 and by day 29 it was normal in both genotypes. However, the splenic

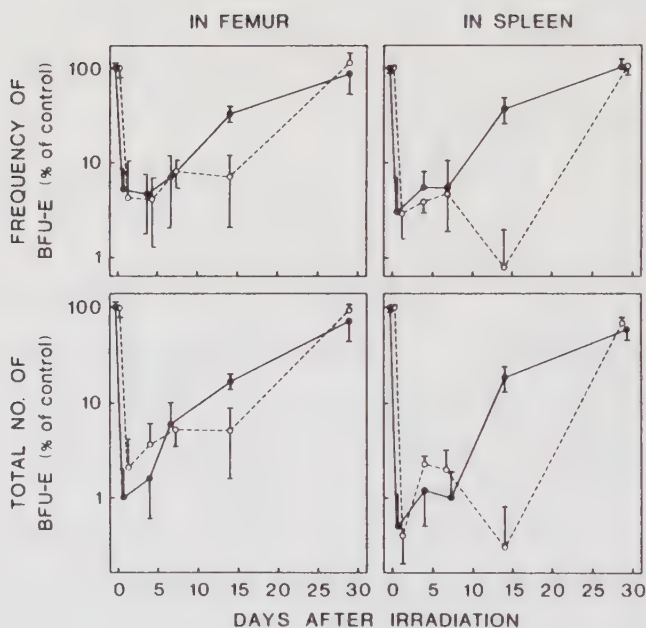


Fig. 20. Patterns of recovery of femoral and splenic BFU-E following 3.0 Gy TBI in wild type (closed symbols, full lines) and homozygous mutant (open symbols, dashed lines) mice. In each group, including non-irradiated controls, BMC and SPC were pooled from 3 mice and each cell suspension was assayed for BFU-E in 5 replicate cultures stimulated with WEHI-3B-CM and EPO (see 5.10.2). Each point with vertical bars represents the mean $\pm$ SD in percentage of control.

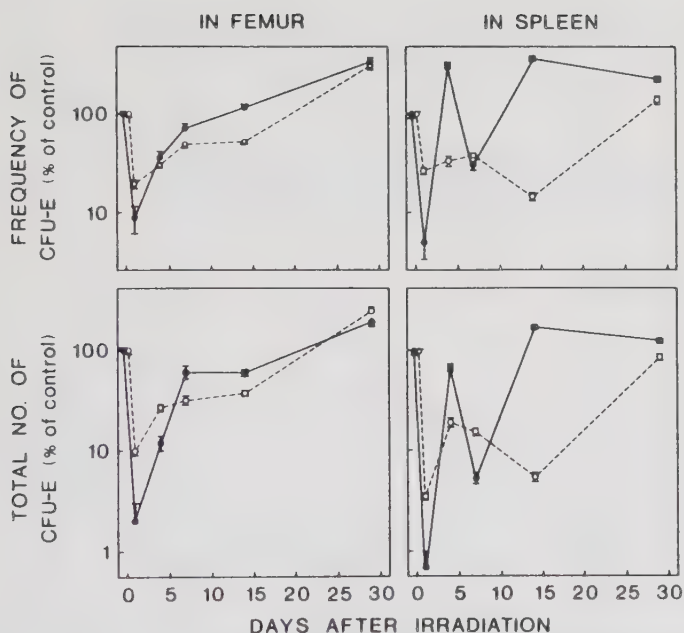


Fig. 21. Patterns of recovery of femoral and splenic CFU-E following 3.0 Gy TBI in wild type (closed symbols, full lines) and homozygous mutant (open symbols, dashed lines) mice. In each group, including non-irradiated controls, BMC and SPC were pooled from 3 mice and each cell suspension was assayed for CFU-E in 5 replicate cultures stimulated with EPO (see 5.10.2). Each point with vertical bars represents mean $\pm$ SD in percentage of control.

Table 15. Femoral and splenic cellularities in wild type (+/+) and homozygous mutant (+*/+*) mice following 3.0 Gy TBI

Days after irradiation	Genotype	No. of cells/femur ($\times 10^{-6}$)		No. of cells/spleen ($\times 10^{-6}$)	
		Irradiated ^a	% of control ^a	Irradiated ^a	% of control ^a
1	+/+	2.7	18.8	21	15.0
	+*/+*	4.5	48.4	106	13.4
4	+/+	3.8	33.9	23	22.8
	+*/+*	7.0	88.6	575	58.7
7	+/+	6.4	84.2	20	17.7
	+*/+*	5.9	65.6	335	42.3
14	+/+	4.8	51.1	58	50.4
	+*/+*	7.0	72.9	385	39.2
29	+/+	8.5	85.0	64	58.7
	+*/+*	8.9	84.8	690	65.2

^aEach irradiated and control value was obtained from bone marrow or spleen cell suspension pooled from 3 mice.

cellularity remained below the normal level even at day 29 in both genotypes.

6.7.4 Observations following 5.0 Gy TBI

The mutants died within 5-6 days when they were irradiated with 5.0 Gy. Following irradiation RBC count, reticulocyte count and spleen weight were examined in wild type and mutant mice.

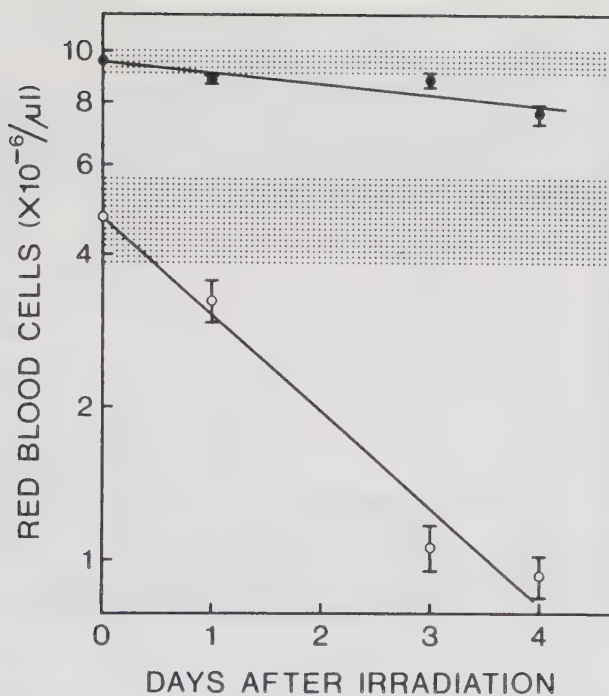


Fig. 22. Red blood cell counts in wild type (closed symbols) and homozygous mutant (open symbols) mice following 5.0 Gy (rate 1.12 Gy/min) TBI. Each point with vertical bars represents mean $\pm$ SD of values from 3 mice examined. The upper and lower shaded areas show the normal ranges in wild type and mutant mice, respectively.

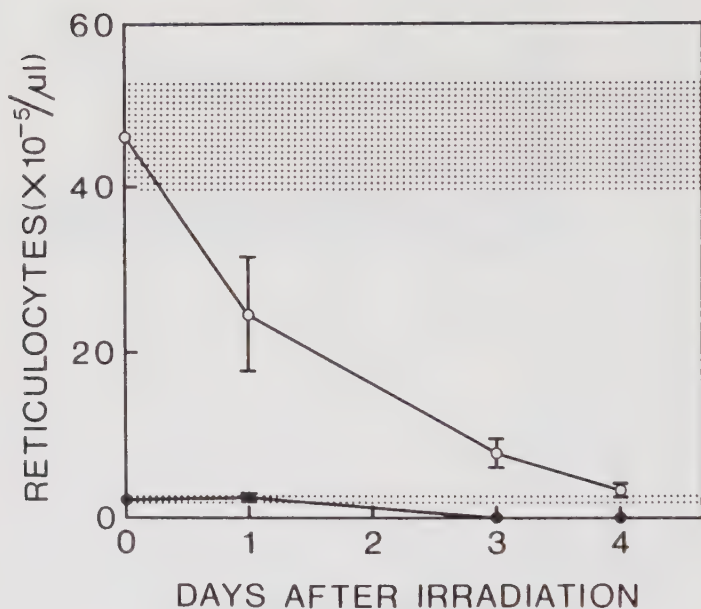


Fig. 23. Reticulocyte counts in wild type (closed symbols) and homozygous mutant (open symbols) mice following 5.0 Gy (rate 1.12 Gy/min) TBI. Each point with vertical bars represents mean $\pm$ SD of values from 3 mice examined. The upper and lower shaded areas show the normal ranges in wild type and mutant mice, respectively.

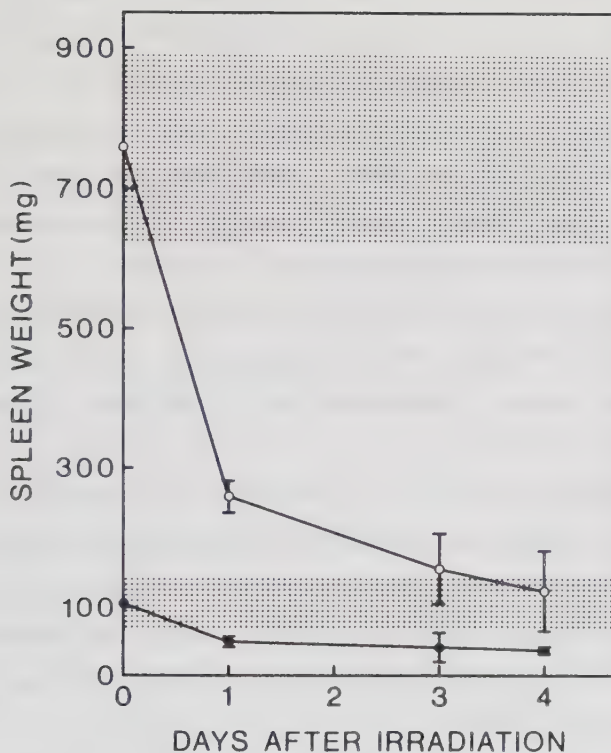


Fig. 24. Spleen weight changes in wild type (closed symbols) and homozygous mutant (open symbols) mice following 5.0 Gy (rate 1.12 Gy/min) TBI. Each point with vertical bars represents mean $\pm$ SD of values from 3 mice examined. The upper and lower shaded areas show the normal ranges in mutant and wild type mice, respectively.

Fig. 22 shows a drastic fall of the RBC count in the peripheral blood of the irradiated mutants. On day 4 the number was about 20% that of non-irradiated controls. The $t_{1/2}$ was calculated to be 1.6 days. On the contrary, in irradiated wild type mice there was but a little dip up to day 4 and it was normalized afterwards (data not shown).

In mutants, the percentage of reticulocytes decreased to 7.4% of control values on day 4. In wild type mice, there was no detectable reticulocyte on days 3 and 4 (Fig. 23).

The spleen weights in the irradiated mutants decreased more rapidly than in the wild type. After 4 days the spleen weight was 16% of the untreated control in the mutants, while it was 34% in the wild type (Fig. 24).

Histological investigations of mutants fixed in formalin just after death revealed that the erythropoiesis was almost absent in the spleen and the bone marrow was almost aplastic with a poor cellularity and some macrophages showing phagocytized erythrocytes. Moreover, the mutants had lung-oedema. Other organs appeared anemic without any lethal changes.

6.7.5 Discussion

From the series of observations made with irradiated wild type and homozygous mutant mice, the great difference of radiosensitivity could be analyzed. The low value of $LD_{50/30}$ in the mutants was the first indication of their higher radiosensitivity. Other mouse strains with macrocytic anemia also showed low $LD_{50/30}$ values (Russel, 1979). The cause of increased radiosensitivity in W/W^v mice ($LD_{50/30}$ 3.65 Gy) turned out to be a delay in regeneration of the erythropoietic tissue

(Russel et al., 1963). The cause of extreme radiosensitivity of Sl/Sl^d mice (LD_{50/30} 1.50 Gy) has been attributed to the inhibition of proper proliferation and differentiation of hemopoietic stem and erythroid progenitor cells by the defective stromal environment (Russel, 1979).

The D₃₇ values for hemopoietic stem and progenitor cells were comparable between the two genotypes (Figs. 12-15). D₃₇ values obtained by different authors have been reviewed by OKunewick (1985). The D₃₇ value obtained by Till and McCulloch (1961) in the C3H strain was slightly higher (1.15 Gy) than the present values (0.90 and 0.98 Gy) in wild type and mutant mice. A comparable value of D₃₇ for GM-CFC (1.55 Gy) has been observed in ddY strain mice (Aizawa et al., 1984). BFU-E (D₃₇ was 1.20 Gy) in the BDF 1 strain of mice, observed by Aizawa et al. (1984) were less radiosensitive than those of the present genotypes.

Regeneration of CFU-S (Fig. 17) in both femurs and spleens of both genotypes after irradiation with 3.0 Gy showed a 'post-irradiation dip' which persisted much longer in the spleens than in the femurs. This phenomenon has also been observed by Guzman and Lajtha (1970), however, not to such a high degree as in the present genotypes. In femurs of mutants, regeneration of CFU-S was slower than in the wild type. The possible reason may be a post-irradiation damage followed by more differentiation than self-renewal to compensate for the hemolytic disease.

The regeneration curves of RBC and reticulocytes in mutants irradiated with 3.0 Gy (Fig. 16) show that the percentage of reticulocytes is in the range of pre-irradiation value for the whole period. However, the RBC count falls to less than 50% of the control value (day 4). On day 7 the RBC count is normal, but a drop is again observed on day 14, which correlates with the drop of BFU-E both in femurs and in spleens,

indicating insufficient commitment of progenitors in the irradiated mutants. The CFU-E in femurs of both genotypes remained at a higher level, about twice the normal level until day 30, although the cellularity was normal by that time. The same type of result was found by Beran (1973) in the morphologically recognizable erythroid lineage of NMRI mice.

After irradiation with 3.0 Gy in femurs the course of regeneration of CFU-E was comparable in both genotypes (Fig. 21). The CFU-E in the spleen of wild type showed an overshooting of frequency by day 4 and then a fall followed by a rise to more than 100 percent. Although the degrees of oscillation were not the same, comparable curves for morphologically recognizable erythropoiesis have been observed by Beran (1970) in the murine spleen after irradiation with 3.0 Gy. In the mutants, slight oscillation in the regenerating process was observed and, in general, the regeneration was delayed. The splenic cellularity in both genotypes was not normalized by day 29, although the spleen weight was in the normal range. In order to give a correct interpretation, more data, especially histological investigations, are required.

The regeneration pattern of GM-CFC in the wild type femur is comparable to the data shown by Aizawa et al. (1984) in ddY mice. The present mutants, on the other hand, showed an oscillating curve. The slow process of regeneration may be due to a lack of sufficient recruitment from the CFU-S compartment, or to differentiation pressure, or both.

During the initial phase of post-irradiation the splenic progenitors (besides BFU-E) in the wild type were depleted more quickly than in the mutants. The spleens of the mutants have a larger reservoir of progenitors than the wild type spleens. However, it was used up within a week

and it can be assumed that due to slow commitment from the hemopoietic stem cell level the values remained low. It is also suggestable that in the mutants less number of progenitors are put into cycle than in the wild type.

Observations following irradiation with 5.0 Gy TBI explained the reason for the extreme radiosensitivity in the homozygous mutants. The erythrocytes of the mutants have a very short life span and they die within 1-2 days. So after 4 days the mutants had only 20% of their total RBC (Fig. 22); the total reticulocyte count was decreased as well (Fig. 23). Following irradiation there is a normal lack of supply of reticulocytes as most of the stem and progenitor cells are depleted, and the mutants die as a consequence of severe anemia. This notion was supported by the autopsy findings. The wild type erythrocytes have a life span of about 43 days, which is long enough to prevent severe anemia during the phase of erythropoietic recovery.

6.8 Bone marrow transplantation

Bone marrow transplantation experiments were performed to answer the following questions:

- 1) Is the hemolytic disease transplantable, i.e., do the mutant hemopoietic stem cells possess a sufficient repopulating capability?
- 2) Is it possible to cure or alleviate the hemolytic anemia through transplantation of healthy bone marrow cells, i.e., does the micro-environment of the mutant support normal hemopoiesis?

6.8.1 Transplantation of mutant bone marrow into wild type mice

Wild type mice were lethally irradiated (8.0 Gy) and subsequently reconstituted with 3×10^6 mutant BMC. Irradiated (8.0 Gy) wild type mice reconstituted with wild type BMC served as controls. A schematic diagram of the experimental design is shown in Fig. 25. The chimeras were examined for RBC count, reticulocyte count, LDH activity, spleen weight and radiosensitivity. Three chimeras of each group, i.e., wild type/mutant (+/+I+*/+*) and wild type/wild type (+/+I+/+), were examined at weeks 1, 2, 3, 4, 5, 7, 9, 11, 13 and 15 after transplantation.

The RBC count in +/+I+*/+* chimeras showed a faster change than other characteristics and was reduced to the level of the mutants already at week 5 (Fig. 26). The RBC count in +/+I+/+ chimeras showed a little dip at the first week, but later it remained at the normal level of wild type.

The reticulocyte percentage in the +/+I+*/+* chimeras gradually increased, and the LDH activity gradually decreased (Figs. 27 and 28); both were in the range of untreated mutant values at week 9. The

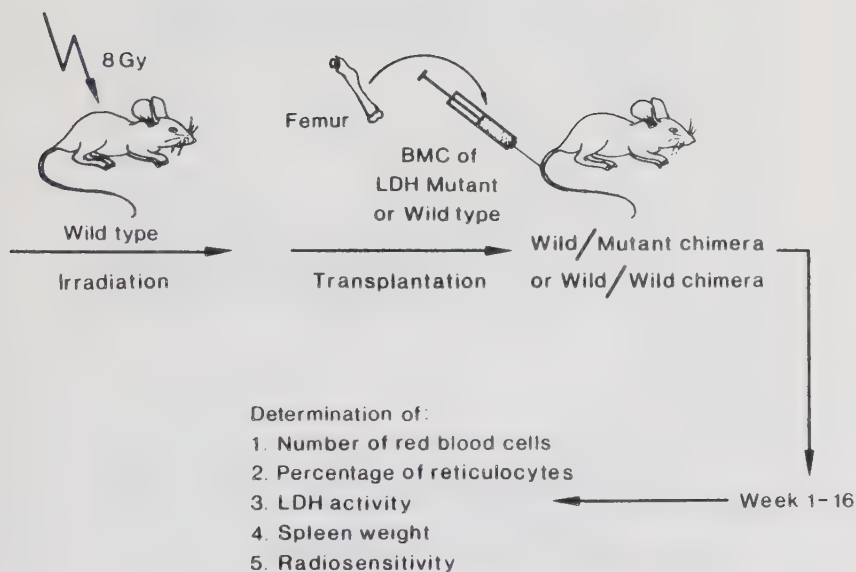


Fig. 25. Schematic representation of the experimental design used for establishment of wild type/homozygous mutant and wild type/wild type chimeras.

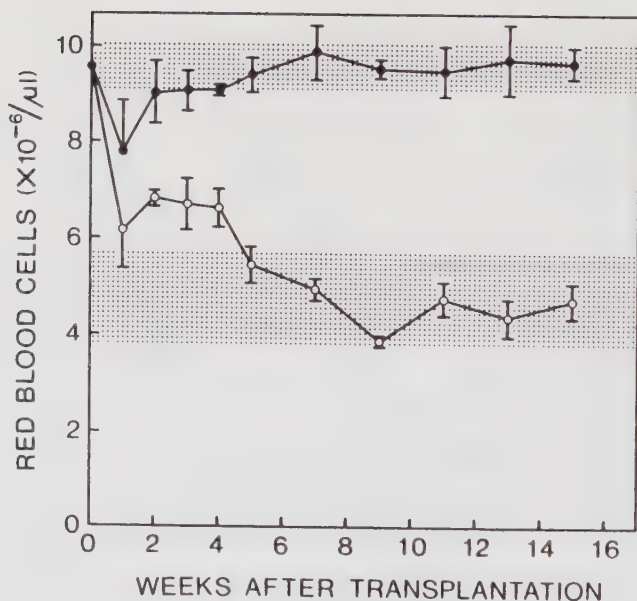


Fig. 26. Red blood cell counts in peripheral blood of wild type/homozygous mutant (open symbols) and wild type/wild type (closed symbols) chimeras generated by 8.0 Gy TBI. Each point with vertical bars represents the mean $\pm$ SD of 3 chimeras examined. Upper and lower shaded areas show the normal ranges in wild type and mutant mice, respectively.

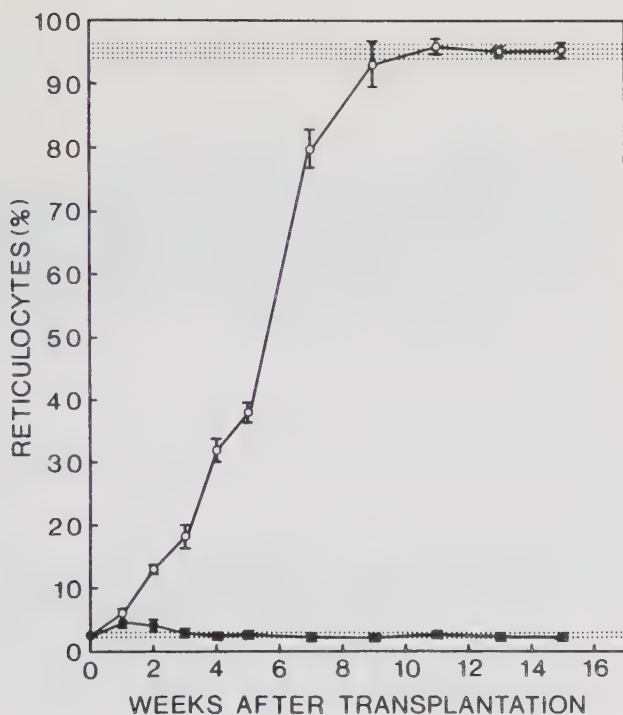


Fig. 27. Reticulocyte percentage in peripheral blood of wild type/homozygous mutant (open symbols) and wild type/wild type (closed symbols) chimeras generated by 8.0 Gy TBI. Each point with vertical bars represents mean $\pm$ SD of 3 chimeras examined. Upper and lower shaded areas show the normal ranges in mutant and wild type mice, respectively.

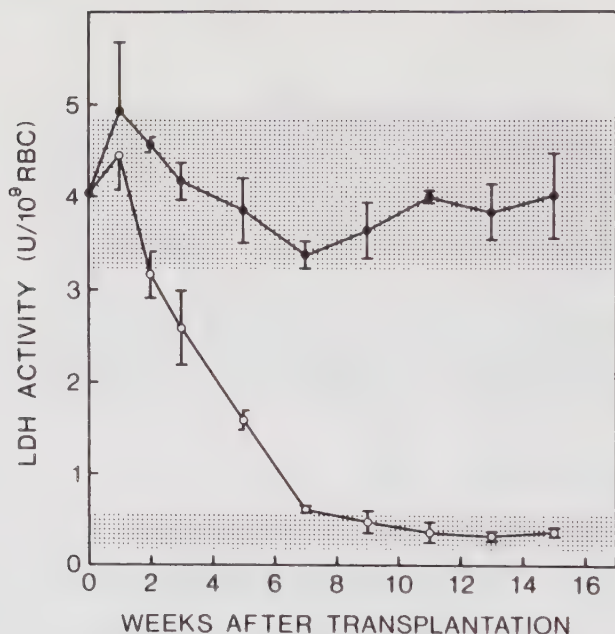


Fig. 28. LDH activity in peripheral blood of wild type/homozygous mutant (open symbols) and wild type/wild type (closed symbols) chimeras generated by 8.0 Gy TBI. Each point with vertical bars represents mean $\pm$ SD of 3 chimeras examined. Upper and lower shaded areas show normal ranges in wild type and mutant mice, respectively.

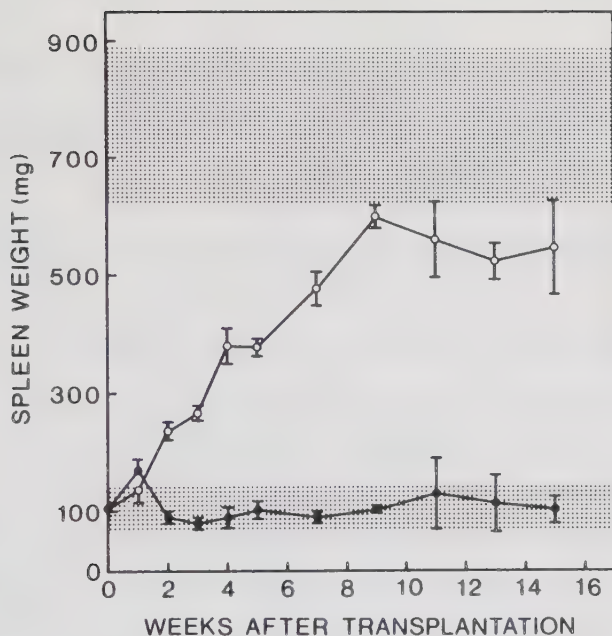


Fig. 29. Spleen weight in wild type/homozygous mutant (open symbols) and wild type/wild type (closed symbols) chimeras generated by 8.0 Gy TBI. Each point with vertical bars represents mean $\pm$ SD of 3 chimeras examined. Upper and lower shaded areas show normal ranges in mutant and wild type mice, respectively.

control data were in the normal range of wild type.

Like other characteristics in +/+I⁺/*/* chimeras the spleen weight also increased (Fig. 29). Although it remained in the lower range of mutant spleen weight, it was significantly different from the control values.

Ten chimeric mice from each group were tested for radiosensitivity by irradiating with 5.0 Gy, at which dose the mutants die within 5-6 days. +/+I⁺/*/* chimeras died on day 4 or 5.

6.8.2 Transplantation of wild type bone marrow into mutant mice

Since the homozygous mutants were extremely radiosensitive, it was not possible to establish a mutant/wild type (+/*/*I⁺/+) chimera following a scheme converse to the above. Lethally irradiated (8.0 Gy) mutant mice subsequently reconstituted with 30×10^6 wild type BMC died within 6 days due to a rapid red cell depletion. In the next experiment mutants were irradiated sublethally (3.0 Gy) and 9×10^6 wild type BMC were transfused per mouse. Four chimeras examined after 4 weeks showed no change in the RBC count, but did show a decreased reticulocyte percentage ($3.7 \pm 2.4\%$) as well as an increased LDH activity ranging from 0.57-4.86 IU/ 10^9 RBC. In two chimeras the spleen weight was quite low (mean 342 mg) and the other two exhibited weights like in untreated mutants. But these changes were only temporary; no difference from untreated mutants was observed after 10 weeks.

Non-irradiated mutants transplanted with 9×10^6 wild type BMC showed no changes in peripheral blood values or spleen weight.

In the next experiment mutants were irradiated with 4.0 Gy (at which dose a fraction of the irradiated mice dies) and reconstituted with

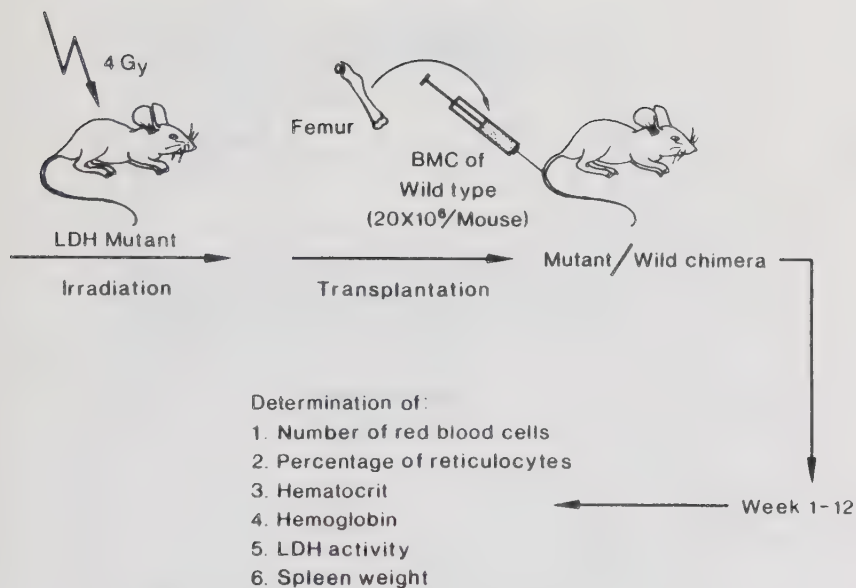


Fig. 30. Schematic representation of the experimental design used during establishment of homozygous mutant/wild type chimeras.

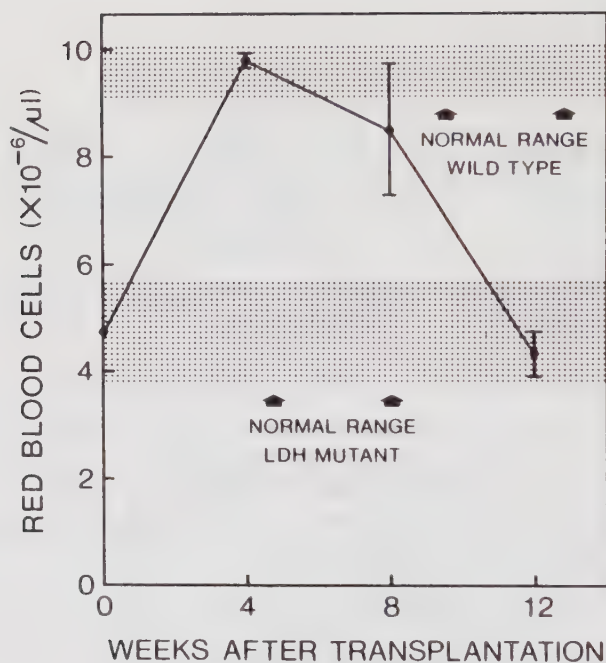


Fig. 31. Red blood cell count in peripheral blood of homozygous mutant/wild type chimeras generated by 4.0 Gy TBI. Each point with vertical bars represents mean $\pm$ SD of 3 chimeras examined. The shaded areas represent normal ranges.

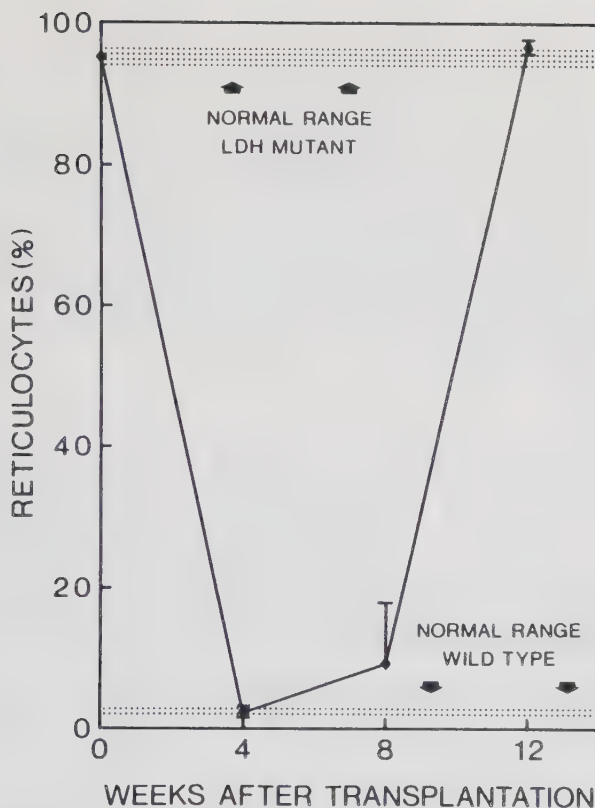


Fig. 32. Reticulocyte percentage in peripheral blood of homozygous mutant/wild type chimeras generated by 4.0 Gy TBI. Each point with vertical bars represents mean $\pm$ SD of 3 chimeras examined. The shaded areas show normal ranges.

Table 16. Hematocrit and hemoglobin values in peripheral blood of homozygous mutant/wild type chimeras^a

Weeks after transplantation	Hematocrit (%)	Hemoglobin (g/dl)
4	46.2 ± 1.6	15.5 ± 0.7
8	38.0 ± 7.3	13.2 ± 2.6
12	34.3 ± 2.1	8.8 ± 0.8

^aMutant mice irradiated with 4.0 Gy were subsequently reconstituted with 20×10^6 wild type BMC (in a volume of 0.5 ml per mouse). Data are expressed as mean ± SD of values from 3 chimeras.

20×10^6 wild type BMC per mouse (Fig. 30). The +*/+*I/+ chimeras were examined at 4, 8 and 12 weeks after transplantation for RBC count, reticulocyte percentage, LDH activity and spleen weight (Figs. 31-34). Figs. 31 and 32 show the RBC and reticulocyte counts, respectively. After 4 weeks the values in the chimeras were fully in the normal range of wild type mice. Hematocrit and hemoglobin values (Table 16) as well as LDH activity (Fig. 33) were also at the level of the wild type mice. After 8 weeks the RBC count was below and the reticulocyte count above the normal range of the wild type mice. Individual LDH values tended towards the range of heterozygous mutants, which is in between those of wild type and homozygous mutant mice. At week 12 all the parameters were in the range of the mutants. Contrary to the other values, the spleen weight in the chimeras at weeks 4 and 8 was in a range between wild type and mutant values, indicating only some loss in cellularity (Fig. 34).

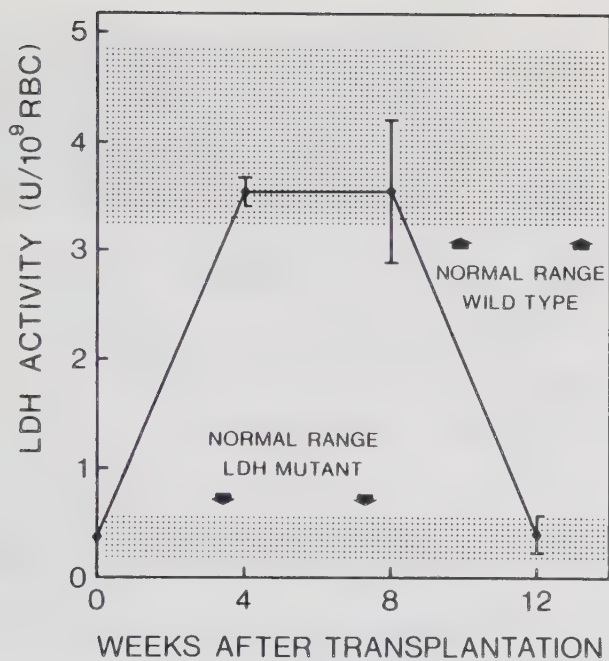


Fig. 33. LDH activity in the peripheral blood of homozygous mutant/wild type chimeras generated by 4.0 Gy TBI. Each point with vertical bars represents mean $\pm$ SD of 3 chimeras examined. The shaded areas show the normal ranges.

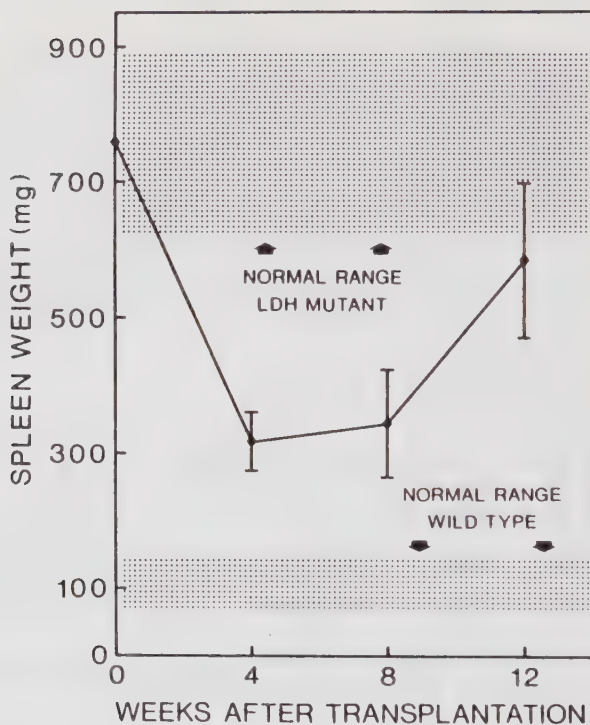


Fig. 34. Spleen weight in homozygous mutant/wild type chimeras generated by 4.0 Gy TBI. Each point with vertical bars represents mean $\pm$ SD of 3 chimeras examined. The shaded areas show the normal ranges.

6.8.3 Transplantation of wild type bone marrow into lethally irradiated mutant mice combined with blood transfusions

Since the preceding investigations did not lead to a permanent establishment of mutant/wild type chimera, another experiment was carried out, the schematic diagram of which is shown in Fig. 35. The mutants were transfused with wild type blood 3 days before, and 7 and 11 days after lethal irradiation with 8.0 Gy. Blood transfusion (more than 5.5×10^9 RBC/mouse) was performed to prevent the severe anemia of the mutants following lethal irradiation. On day 3 lethally irradiated mutants were reconstituted with 20×10^6 wild type BMC per mouse. They were observed at week 4, 8, 12 and 16 after bone marrow transplantation and measurements of RBC and reticulocyte count, hemoglobin and LDH activity in peripheral blood were performed (Table 17). Five chimeras were tested at each time point. All the characteristics examined were in the normal range of wild type except that the RBC count was slightly above normal until week 12. This may be an effect of extensive blood transfusion. Spleen weights were not measured, in order to keep the chimeras for a long term observation. The chimeras appeared to have a normal life span. Autopsy and histological observations, 9 months after transplantation, revealed no differences between the $+^*/+^*I/+$ chimeras and wild type mice. 17 weeks after transplantation, 20 chimeras were irradiated with 5.0 Gy and all of them survived the irradiation.

6.8.4 Discussion

Lethally irradiated (7-9 Gy) mice die within 9-18 days due to severe WBC and platelet depletion. Bone marrow grafts from healthy donors

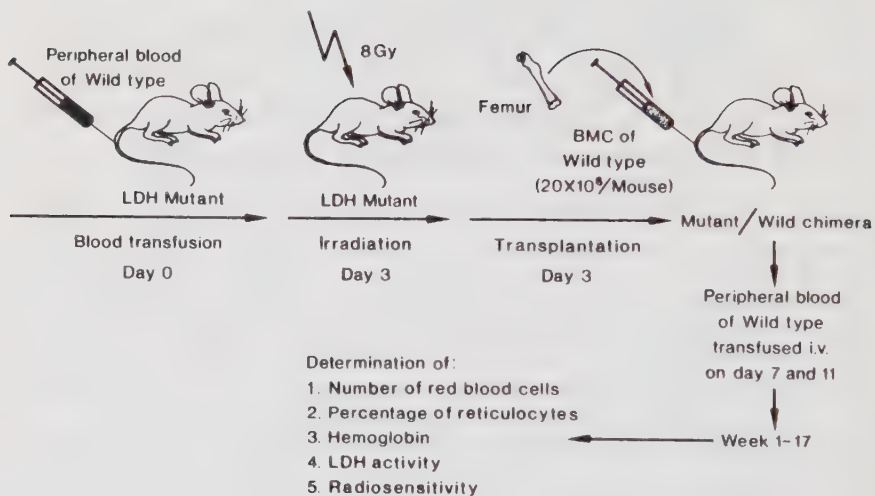


Fig. 35. Schematic diagram of the experimental procedure used to establish a permanent homozygous mutant/wild type chimera.

Table 17. Peripheral blood values in permanent homozygous mutant/wild type chimeras^a

Weeks after transplantation	RBC ($\times 10^{-6}/\mu\text{l}$)	Reticulocytes (%)	Hb (g/dl)	LDH (IU/ 10^9 RBC)
4	10.73 $\pm$ 0.74	2.3 $\pm$ 0.6	16.8 $\pm$ 1.3	3.38 $\pm$ 0.27
8	10.32 $\pm$ 0.88	2.1 $\pm$ 0.6	15.7 $\pm$ 2.0	3.54 $\pm$ 0.49
12	11.32 $\pm$ 0.49	2.3 $\pm$ 0.4	n. d. ^b	2.93 $\pm$ 0.20
16	9.76 $\pm$ 0.63	2.4 $\pm$ 0.3	15.8 $\pm$ 0.9	3.42 $\pm$ 0.26

^aMutant mice were transfused i.v. with wild type blood (more than 5.5×10^9 RBC/mouse) 3 days before, and 7 and 11 days after lethal irradiation with 8.0 Gy and reconstitution with 20×10^6 wild type BMC (in a volume of 0.5 ml per mouse). Data are expressed as mean $\pm$ SD of values obtained from 5 chimeras.

^bn. d. = not determined

could assure survival of those lethally irradiated animals (Bond et al., 1965; Micklem and Loutit, 1966). This phenomenon has already been observed by Lorenz et al. in 1951. In the first experiment of the present transplantation series, lethal irradiation of wild type mice followed by transplantation with mutant BMC resulted in a permanent +/+I+*/+* chimera with all the hemopoietic features of the mutants. Through lethal irradiation all the host pluripotent stem cells could be eradicated, providing space for the transfused mutant stem cells to colonize and proliferate in the recipients' hemopoietic sites.

Transplantation of BMC in a non-irradiated host was reported by Micklem et al. (1968) for the first time. Following transplantation of 20×10^6 syngeneic BMC (approx. 6% of total hemopoietic cells in the normal mouse, Chervenick et al., 1968) an average of only 2.5% of donor mitoses in the recipients' marrow were found and in many animals no donor cells were identified (Micklem et al., 1968). In similar studies

(Takada et al., 1970; Takada et al., 1971; Brecher et al., 1979) 2% or less donor mitoses were found in most of the recipients. The proposed explanation was that irradiation was necessary to destroy the host's stem cells and create empty domains (Malony et al., 1978), proliferative sites (Micklem et al., 1968) or niches (Schofield, 1978) for the colonization and proliferation of the syngeneic donor cells. Following massive bone marrow transplantation (200×10^6 BMC/mouse, which was estimated to be 66% of the total marrow cells in the non-irradiated normal mouse), however, 16-40% cells of the recipients' marrow and peripheral blood were found to be of donor origin (Brecher et al., 1982; Brecher et al., 1985). In another publication, elevation of the recipients' CFU-S after such a transfusion has been reported and it has been suggested that the additional number of stem cells were of donor origin (Cronkite et al., 1985). However, this elevated level existed only for two months. The authors supposed that some regulation at the stem cell level is responsible for this fact and referred to the postulation of humoral regulation of the stem cells by Lajtha (1979).

Non-irradiated mutants transplanted with 9×10^6 wild type BMC showed no change in peripheral blood values or spleen weight. It can be concluded that this number of BMC was very low in comparison to those used by Brecher et al. (1982, 1985) and Cronkite et al. (1985) to produce any visible effect. Since in this way it was impossible to achieve a permanent chimerism, other methods were applied. In W/W^v mice, however, the hereditary macrocytic anemia could be cured by bone marrow transplantation without prior host-irradiation (Bernstein and Russel, 1959). In the W mutant, defects in self-renewal and differentiation of the hemopoietic stem cells are responsible for a very low number of stem cells (Merchav et al., 1985), and normal stem cells have

an advantage over the mutant ones (Schofield, 1978).

The reticulocyte count and LDH activity in $+*/+*I/+$ chimeras, produced after irradiation with 3.0 Gy, suggest that some of the donor CFU-S and/or progenitors proliferated and differentiated. But they were overgrown very fast by the regenerating host CFU-S. $+*/+*I/+$ chimeras produced after irradiation with 4.0 Gy show all the peripheral blood phenotypic expressions of the wild type (Fig. 35, Table 17). This is temporary and no longer observable at the week 8. The changes with time are similar to those observed by Cronkite et al. (1985), namely, a restoration of the pre-transfusion condition within two months. Although these two experiments are not quite comparable, in both cases the same mechanism may have been operative, i.e., some regulation at the stem cell level favoring the stem cells of the host.

Contrary to the above situations, observed in radiation chimeras of LDH mutants after sublethal irradiation, the α -thalassemic mice ($Hbath/+$) (Russel et al., 1976) could be cured of their hereditary microcytic anemia by irradiation with 3.0 Gy and transplantation with 10^6 $+/+$ BMC (Wagemaker et al., 1986). In the bone marrow, the space created by sublethal irradiation was sufficient for the establishment of a permanent chimerism, resulting from a selective advantage of normal stem cells in the erythroid differentiation pathway.

In the present mutant, permanent chimerism could only be achieved by combining bone marrow transplantation with blood transfusion (Fig. 35). A complete depletion of the host's pluripotent stem cells was necessary for this purpose. The death of the mutants after irradiation with 8.0 Gy in spite of a high dose of bone marrow transplantation (6.8.2) indicates that the number of transplanted BMC was not the most critical factor for the animals' survival. Since blood transfusion prevented

early death of the mutants during the phase of expansion of the newly colonized stem cells, rapid RBC depletion was identified as the factor limiting survival of the irradiated and transplanted mutant mice.

There are different opinions regarding the transplantability of the hemopoietic microenvironment via an intravenous route. The hemopoietic microenvironment is composed of several elements, such as fibroblasts, macrophages and endothelial cells. From long-term bone marrow cultures one group of investigators (Friedenstein et al., 1978; Bentley et al., 1982; Lennon and Mickelm, 1986) has shown the host origin of fibroblastoid stromal cells, while the endothelial cells were of donor type. Contrary to these observations, Piersma et al. (1983) and Marshall et al. (1984) have reported that a fraction of fibroblastoid stromal component or epithelial cells was of donor origin. Due to these controversial observations among different investigators, no conclusion concerning the origin of the hemopoietic microenvironment in the present set of transplantation experiments could be drawn.

CHAPTER 7. GENERAL DISCUSSION

The investigations of the present project were performed in order to analyze various aspects offered by the model of hemopoietic stress in the lactate dehydrogenase mutant mouse. The following comprehensive review of the results described in detail in Chapter 6 should make it easier to understand the connections between the various experiments.

Reticulocytosis and splenomegaly are common symptoms for an elevated red cell production in hemolytic anemia. In the LDH mutant the enzyme deficiency and hemolytic anemia are inherited simultaneously, leading to the supposition that the anemia is a consequence of the low enzyme level in the red blood cells. In the erythrocytes, the only metabolic pathway of gaining ATP is glycolysis (Embden-Mayerhof pathway); any change of the enzymes taking part in that pathway may shorten the erythrocyte life span as it has been observed in several hemolytic anemias in man (Wintrobe et al., 1981). In the mutants, the low RBC count (50% that of normal) and the high reticulocytosis (Table 3) indicate a very short erythrocyte life span. This notion is supported by the rapid red cell depletion following irradiation with only 5.0 Gy (see 6.7.4). In other inherited hemolytic anemias of mice the erythrocyte life span has also been reported to be very short (≤ 1 day) (Bernstein, 1980).

Quantification of the erythroid nucleated and recognizable as well as stem and progenitor cells reveals only indirect effects of the LDH deficiency along the erythroid pathway of differentiation. The early destruction of the erythrocytes causes an erythropoietic stress. To cope with this extra demand the pool of the more mature erythroid precursors is greatly increased (Tables 8, 10), as a consequence of

which the spleen is enlarged. On the contrary, the pool of CFU-S, GM-CFC and BFU-E in the mutants was almost the same as in the wild type mice. Comparable observations in mice and rats with protracted anemia induced by phenylhydrazine have been made by several authors (Rencricca et al., 1970; Hara et al., 1976; Hodgson et al., 1976). In the hemopoietic compensation of the LDH mutants the possibility for the CFU-S to be expanded remains open, without necessarily causing additional changes in the physiological flux through the individual compartments. Quite remarkably, in this compensation an elevated rate of proliferation at the stem cell level could not be documented (Table 14). It has been calculated from the total body content of CFU-E and BFU-E that 4 additional divisions should occur along the erythroid pathway between BFU-E and CFU-E in the mutants (see 5.11). In the mutants, the cell cycle time of the erythroblasts most likely is shortened as indicated by the reduced DNA synthesis time (Fig. 7) in the basophilic and polychromatic erythroblast compartments of the spleen and the increased labeling index (also observed by Dörmer (1981) in phenylhydrazine treated rats).

The spleen of the homozygous mutant mouse plays an important role in the hemopoietic compensation as evident from its contribution to total hemopoiesis (Table 11). However, the long survival of the mutants after splenectomy calls this fact into question. The splenectomized mutants were constantly more anemic than untreated ones, suggesting indeed a significant, however, dispensible role of the spleen. Extramedullary hemopoiesis in the liver was observed in the present mutant only after splenectomy, whereas mutants with spherocytic, hemolytic anemia have been reported to possess liver hemopoiesis (Unger et al., 1983).

Homozygous mutants showed an extremely high radiosensitivity as de-

signated by the value of $LD_{50/30}$ (7.3 Gy and 4.4 Gy for the wild type and mutant mice, respectively). Hemopoietic pluripotent stem cells and progenitors of the two genotypes showed comparable radiosensitivity and therefore could not be traced with the increased radiosensitivity in the mutants. Moreover, delayed recovery of CFU-S in the femur and of BFU-E and CFU-E in spleen following sublethal irradiation (Figs. 18, 20, 21) in the mutants was not marked enough to serve as an explanation. The cause of this abnormality was found to be a very short erythrocyte life span. Comparable data in literature showing similar causes of radiosensitivity in anemic mice are not available.

The last biological question addressed in Chapter 3 was answered by bone marrow transplantation experiments. The clinical manifestations of the hemolytic disease of homozygous mutants were transplantable into wild type mice through bone marrow grafting (Figs. 25-29). Transplantation of wild type bone marrow into sublethally irradiated mutant mice produced a transient chimerism with some peripheral blood characteristics of the wild type mice, but failed to produce any permanent effect (Figs. 30-34). In contrast, it was possible to cure the α -thalassemia in mice by adopting a similar procedure (Wagemaker et al., 1986). Unlike in the α -thalassemic mice, in the present mutant it is apparent that host stem cells possess an advantage in the host hemopoietic sites (Micklem et al., 1968; Malony et al., 1978; Schofield, 1978) over the donor ones. A high dose of bone marrow graft could not save the lives of the mutant mice irradiated with 8.0 Gy. However, they could be cured of their disease by application of bone marrow transplantation combined with transfusion of wild type RBC (Fig. 35, Table 17). This, again, emphasizes that the cause of increased radiosensitivity in the mutants is the early death of the short-lived erythrocytes in the absence of

sufficient new production.

The following conclusions can be drawn from the above discussion:

1. The red blood cells of the mutants appear to be directly affected by the LDH deficiency resulting in a very short erythrocyte life span.
2. The hemolytic anemia is compensated for primarily by an enlargement of the more mature erythroid precursor pool, and to some extent, by a shortening of the erythroblast maturation time.
3. The spleen in the homozygous mutant largely contributes to total hemopoiesis, however, it is not indispensable for the animal's survival.
4. The homozygous mutants are extremely radiosensitive.
5. Through bone marrow transplantation, it is possible to transfer the hemolytic disease to the wild type. Vice versa, mutants are cured by wild type marrow.

SUMMARY

The codominant mutation of the muscle type of lactate dehydrogenase (LDH-A) enzyme is manifested by severe hemolytic anemia in the homozygous mutant mice. The remarkable characteristics of the mutants are: a low number of red blood cells (RBC), high reticulocytosis, and splenomegaly. The high rate of red cell loss is compensated for to such a degree that the mutant mice have a largely normal life span and fertility. The erythropoietic compensatory mechanism in the mutants as well as changes in hemopoiesis due to external stress (e.g., irradiation) and the repopulating capability of the stem cells of one genotype in the hemopoietic environment of the other were analysed with the help of the investigations summarized below. Except for a low LDH activity -- about 50% that of the wild type -- the heterozygous mutants showed almost no difference from the wild type.

In order to assess the level at which compensation takes place, quantification of the morphologically recognizable precursors was carried out. The fraction of the erythroid population was elevated in the bone marrow (32%) and especially in the spleen (66%) of the homozygous mutants in comparison to that of the wild type mice (19% and 9%, respectively). In the mutants, these values correlated with the enlarged pool of late erythroid progenitor cells (CFU-E): the total body content of CFU-E was about 18 times higher and 91% of the total CFU-E were found in the spleen. The pool of early erythroid progenitors (BFU-E) was slightly increased in the mutants, whereas that of pluripotent hemopoietic stem cells (CFU-S) and granulocyte-macrophage colony-forming cells (GM-CFC) were comparable in both genotypes.

It is apparent from the contribution of the spleen to total hemo-

poiesis that it is an organ of high importance for the mutants. On the other hand, the enlarged spleen may also be responsible for red cell sequestration. It was interesting to note that the mutants survived for at least 652 days following splenectomy, in spite of the fact that they had an RBC count of only about 25% of the untreated mutants. The results indicate that the spleen is not indispensable for the survival of the mutants and splenectomy does not abrogate the hemolytic disease.

In a state of hemolytic anemia it is quite conceivable that the number of one or the other erythropoietic progenitor cell type in S-phase is increased in order to cope with the body's demand for mature erythrocytes. In the proliferation kinetic studies no increase in the number of mutant hemopoietic cells (CFU-S, GM-CFC, BFU-E or CFU-E) in the S-phase was found. However, a reduced DNA-synthesis time was estimated in the morphologically recognizable erythroid population, namely, basophilic and polychromatic erythroblasts.

The homozygous mutants were irradiated in order to estimate their capacity to cover an external stress on hemopoiesis. First of all, the mutants were found to be extremely radiosensitive. Lethal dose_{50/30} (LD_{50/30}) was 4.4 Gy for the mutants as compared to 7.3 Gy for the wild type mice. Mutants irradiated with 5.0 Gy died within 5-6 days due to severe anemia. The cause of the increased radiosensitivity was found to be the very short life span (approximately 1 day) of the erythrocytes. The radiosensitivity at the stem and progenitor cell level was comparable in both genotypes.

Bone marrow transplantation experiments showed that the characteristic features of the homozygous mutants could be transferred into the wild type mice, and thereby it was possible to constitute permanent wild type/homozygous mutant chimeras. However, establishment of a per-

manent homozygous mutant/wild type chimera was not possible simply by host-irradiation (in order to destroy the stem cells) and subsequent bone marrow grafting. The reason behind it was a rapid red cell depletion following irradiation. When the mutants were transfused with normal blood before and after lethal irradiation, they achieved normal radiosensitivity. In this way, permanent mutant/wild type chimeras were produced.

It can be concluded that the inherited deficiency of the enzyme LDH-A, which predominates in the RBC, causes a very short life span (1 or 2 days) of these cells. This view is supported by the rapid decrease of the RBC count in the mutants after irradiation with 5.0 Gy and by the normalization of the mutants' radiosensitivity following excessive blood transfusion.

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Some portions of this thesis have already been published. The publications are follows:

- Datta T, Dörmer P (1987) Establishment of permanent chimerism in lactate dehydrogenase deficient mutant mouse with hemolytic anemia. *Exp Hematol* (in press).
- Kremer JP, Datta T, Dörmer P (1986) A model of hemopoietic stress in a lactate dehydrogenase mouse mutant with hemolytic anemia. *Blut* 52:

Kremer JP, Datta T, Pretsch W, Charles DJ, Dörmer P (1987) Mechanisms of compensation of hemolytic anemia in a lactate dehydrogenase mouse mutant. Exp Hematol (in press).

CURRICULUM VITAE

Tapati datta was born in Malda, West Bengal, India, in 1956. She completed her secondary education (HSE) at the Holy Child Institute, Calcutta, in 1973. Then she entered the Presidency College, University of Calcutta, and studied botany, zoology and chemistry. In 1977 she received the Bachelor of Science degree (first class) and started for Master of Science in botany with special paper on cytology and cytogenetics. She was awarded her degree (first class) at the end of 1979. From February 1980 to April 1981 she worked at the Center of Advanced Study in Botany (Cell and Chromosome Research), University of Calcutta, as a research fellow of the University Grant Commission.

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